IMPACT OF DIURNAL TEMPERATURE FLUCTUATIONS DURING LARVAL DEVELOPMENT ON ADULT LIFE HISTORY TRAITS AND INSECTICIDE SUSCEPTIBILITY IN TWO VECTORS; *ANOPHELES GAMBIAE* AND *AEDES AEGYPTI.*

by

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ABSTRACT

Impact of Diurnal Temperature Fluctuations during Larval Development on Adult Life History Traits and Insecticide Susceptibility in Two Vectors; *Anopheles gambiae* and *Aedes aegypti*.

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Mosquito breeding habitats are exposed to diurnal fluctuations in temperature that developing mosquito larvae must endure. Despite this, work is lacking on what, if any, influence these fluctuations have on adult traits of epidemiological interest. In this work, cohorts from two geographically isolated strains of *Aedes aegypti* (TH from Thailand and BZ from Belize) and a susceptible (G3) and resistant (AKRON) strain of *Anopheles gambiae*, were exposed as larvae to one of four diurnal temperature range (DTR) treatments from 0°C to 20°C around a mean of 28°C. Increasing DTR reduced egg production in the TH strain, but increased production in the BZ strain of *Ae. aegypti*. For *An. gambiae*, increasing DTR decreased egg production with production ceasing altogether at 20°C DTR.

Pupation rates for the BZ strain of *Ae. aegypti* increased, while those for the TH strain decreased, with increasing DTR. Emergence rates for *Ae. aegypti* were little

affected by DTR. For *An. gambiae*, increasing DTR resulted in decreased pupation rates in the resistant AKRON strain but did not affect the G3 strain. However, increasing DTR reduced emergence rates for G3 mosquitoes but had no effect on the AKRON strain. Female survival rates for *Ae. aegypti* were unaffected by DTR treatment. For males, BZ sucrose survival decreased, whereas, TH sucrose survival increased, with increasing DTR. For *An. gambiae*, sucrose survival increased in the AKRON strain but decreased in the G3 strain with increasing DTR. Blood fed and starved survival rates were unaffected.

In the susceptibility assays, increasing DTR exposure resulted in a four-fold increase in permethrin LD_{95} concentrations for the TH strain but little change for the BZ strain of *Ae. aegypti*. However, for both propoxur and malathion, an increase in DTR to 10°C resulted in a decrease in susceptibility for both strains. For *An. gambiae*, large DTR exposure resulted in a two-fold increase in malathion LD_{95} concentrations for the G3 strain, and a 10-20% increase in the AKRON strain. The larval thermal environment had no significant effect on the susceptibility of either strain to propoxur or permethrin. These differences were not enough to affect efficacy of field application rates. These results suggest that larval thermal exposure can have species specific, and strain specific, effects on adult characteristics.

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CHAPTER 1: General Introduction

THE THERMAL ENVIRONMENT

The ambient temperature at which an organism must function can be considered the organism's thermal environment. Ambient temperature can have a huge influence on physiological processes in cold blooded animals. For instance, warmer temperatures have been shown to increase hemolymph pH in the locust *Schistocerca nitens* (Thunburg) (29) and speed hemolymph acidosis recovery rates in the grasshopper *Melanoplus bivittatus* (Say) (28). Also, ambient temperature is known to be an important constraint on the immune response (12) while small changes in ambient temperature have the ability to influence the outcome of insect-parasite interactions (65). Indeed, Benelli (8) showed that this influence can be carried over to the adult stage as larval exposure to low temperatures reduced immune encapsulation responses in adults.

Ambient temperature has been shown to influence measured life-history traits in vector populations (4, 39, 57) as well as altering disease transmission dynamics. For example, Westbrook et al. (70) showed that rearing *Aedes albopictus* Skuse at 18°C produced larger females that were six times more likely to be infected by Chikungunya virus (CHIKV) than when reared at 32°C. Additionally, Watts et al. (68) showed that, while *Aedes aegypti* (Linnaeus) could become infected with dengue virus (DEN-2) at any temperature from 20°C to 35°C, only those mosquitoes held at 30°C or higher were able to subsequently transmit the virus to monkeys, regardless of infectious dose. However, the influence ambient temperature has on vector/pathogen interactions is not consistent across systems. For instance, higher temperatures increase infection rates of Rift Valley

fever virus in *Culex pipiens* Linnaeus (67) while decreasing infection rates of Eastern Equine Encephalitis virus in *Culex tarsalis* Coquillett (36).

THERMAL VARIATION

The main focus of studies to date examining the influence of the thermal environment on insect systems has concentrated on the role thermoperiodism plays in the yearly cyclical patterns of insect populations. A good deal of this work has documented the influence changing thermal environments have on physiological processes and the induction of diapause, particularly at the low end of insect thermal envelopes (7). The effects of different thermal environments on insect growth, development, physiology, and behavior are well studied (6): however, the influence of diurnal temperature fluctuations on these systems has scarcely been addressed. This paucity of work was pointed out 30 years ago by Beck (6) when he noted that the wealth of experimental data describing the influence of temperature on these processes was almost exclusively derived from rearing conditions with constant temperatures. The role temperature variation in the thermal environment plays in shaping the outcomes of these responses is seldom considered.

The influence of temperature on insect development is usually quantified by the use of degree days, or the number of thermal units accumulated above a base developmental temperature threshold. However, Scriber and Sonke (62) noted that these units are calculated using mean daily temperatures without regard to the daily thermal variance. Comparing post-diapause pupal development in two swallowtail butterflies (*Papilio glaucus* Linnaeus and *P. canadensis* Rothschild& Jordan) they showed that, while developmental rate was largely unaffected, female adult size in *P. canadensis* was smaller with increased thermal variance around the same mean temperature.

Interestingly, this effect was not found in *P. glaucus*, suggesting the influence of thermal variation may be species specific.

THERMAL STRESS

Thermal stress can best be thought of as any shift in the thermal environment that forces an organism to operate outside the optimal temperature range for which its physiological processes are optimized to function under. Thermal stress has been shown to influence the outcome of several phenotypic traits. Imasheva et al. (32) reported a trend towards increased phenotypic variation in thorax length, wing length, number of sternoplueral cheatae, and number of arista bristles in *Drosophila melanogaster* Meigen and *D. buzzatii* Patterson & Wheeler reared under thermal stress. Muturi et al. (47) showed that *Ae. aegypti* larvae reared at 32°C were much more susceptible to infection with Sinbis virus as adults. They also showed that exposure to elevated temperatures during immature development led to a decreased expression of heat shock protein 83 and increased expression of defensin and cecropin in subsequent adults and suggested that this led to gut fauna changes making the mosquitoes more susceptible to infection. Mourya et al. (43) had come to a similar conclusion when they examined the influence thermal stress on *Ae. aegypti* larvae had on adult susceptibility to CHIKV. The only work to date that examines the role larval thermal stress plays in the susceptibility of adults to an insecticide was that of Raghavendra et al. (55) who showed that larval exposure to brief periods of thermal stress resulted in one to three fold increases in adult LT_{50} times to 5% malathion impregnated bed nets.

Diurnal Temperature Range

Variation is characteristic of the thermal environment. This includes shifts in mean temperatures caused by cyclical climactic patterns, such as the el-nino southern oscillation, as well as short-term cyclical changes in ambient temperature caused by solar warming during the day and cooling at night. This daily temperature variation, or diurnal temperature range (DTR), is defined as the difference between the daily maximum and minimum temperatures in a given 24 hour period. Diurnal temperature variation in the soil and air has been found to approximate a truncated sine wave warming phase during the day with a decaying exponential cooling phase during the night (54). This daily variation in temperature has the ability to influence the phenotypic expression of traits. For instance, it is known that phenotypic expression of seasonal eye spot patterns in *Bicyclus* spp. butterflies can be manipulated by rearing larvae under varying constant mean temperatures (35). However, Brakefield and Mazzotta (15) showed that the same effect could be obtained by changing the magnitude of the diurnal temperature range while maintaining the same daily mean temperature. Moreover, these variations more closely resembled field phenotypes than did those reared under constant temperature regimens.

In terms of mosquito biology/physiology it can be argued that short-term daily fluctuations in temperature can influence mosquito life history traits much more drastically than shifts in long-term mean ambient temperature, whether over the course of weeks, months, or years. Long-term shifts provide evolutionary forces a chance to shape the phenotype through continued fine-tuning of optimal life history traits through successive generations. However, exposure to daily thermal variation has the potential to influence the outcome of life history traits of individuals within a single generation.

This could lead to differing outcomes for the same measured traits from intra-generational cohorts exposed to differing conditions (phenotypic plasticity) leading to greater observed variability of these traits in a natural population. As a result, natural populations may be better able to cope with long term shifts in mean temperature than may be presumed based on fitness estimates from lab reared cohorts. Indeed, Long (40) suggested that overall population fitness tends to be greater in environments with more frequent fluctuations in temperature while Beardmore and Levine (5) showed that diurnal temperature fluctuations produce *Drosophila psuedoobscura* (Frolova) larvae with higher viability.

Despite this, the majority of disease vector work to date has focused on the role of static mean temperatures on pathogen-vector systems. However, the influence of daily temperature fluctuations on these systems has only recently begun to be explored. Lambrechts et al (38) showed that *Ae. aegypti* from Thailand were less susceptible to dengue virus infection and exhibited faster mortality at larger DTRs around the same mean ambient temperature. In doing so, they showed that the defining predictive factor for dengue virus transmission in Thailand might not be mean seasonal temperature or rainfall but the size of the DTR around the mean daily temperature. Additionally, Paaijmans et al. (53) showed that fluctuation in diurnal temperatures reduces the impact of increasing mean temperatures when using a thermodynamic model for malaria development (model parameters 18-28°C) in the mosquito vector. Specifically, they showed that diurnal temperature fluctuations around means less than 21°C slow parasite development while fluctuations around means greater than 21°C increases parasite development. Expanding on this model, Paaijmans et al (50) showed that, at the model

extremes, diurnal temperature fluctuations make transmission possible at lower mean temperatures than expected while reducing transmission below what is expected at higher mean temperatures. As a result, current models for predicting malaria transmission risk may underestimate transmission at the fringes of endemic zones, such as in the Highlands of East Africa, while, at the same time, overestimating the risk in warmer portions of endemic zones (50).

THERMAL VARIATION AND MOSQUITO DEVELOPMENT SITES

Field mosquito development sites are exposed to the same daily warming and cooling cycles that adult mosquitoes experience. Adult mosquitoes, however, have the ability to moderate their exposure to daily temperature fluctuations through behavioral avoidance, e.g. responding to changes in microclimactic cues via the search and selection of more suitable resting locations. In contrast, exposure of mosquito larvae to diurnal temperature fluctuations is determined by the thermal characteristics of the development site in which eggs are laid. Daily thermal variation may be minimal for species that breed in large bodies of water but for species that utilize small ephemeral sites, such as small puddles, tire ruts, hoof prints, discarded tires and water holding tanks, daily thermal variation may have a significant influence on developing larvae—especially if these locations receive direct sunlight for any portion of the day.

Several prominent disease vectors routinely utilize such habitats as breeding sites. *Anopheles gambiae* Patton, the principal vector of malaria in much of Africa, is known to utilize anthropogenic breeding sites such as tire ruts, hoof prints, and drainage channels (37, 45) as well as natural sources such as rain pools and burrow pits (45). In deforested areas these sites have been shown to be more productive in terms of adult mosquitoes

produced (44) than those in forested areas, presumably due to the fact that direct exposure to sunlight increases mean water temperatures. Additionally, Paaijmans et al. (52) showed that diurnal temperature variation in standard anopheline field development sites resulted in mean water temperatures 4-6°C higher than the mean ambient temperature. *Aedes aegypti,* the prominent vector of dengue-dengue hemorrhagic fever, is a container breeder that can be expected to be routinely exposed to daily temperature fluctuations during larval development. This species is well known for its utilization of small containers as breeding and immature development sites. Many of these sites, such as discarded tires sitting in the sun, can amplify the magnitude of natural ambient temperature fluctuations due to their low albedo. As a result, a large amount of incident solar radiation is absorbed leading to increases in day time temperature maximums.

THERMAL VARIATION AND MOSQUITO DEVELOPMENT

Given the exposure of some larval mosquito populations to diurnal changes in temperature, it is surprising that the effect of this exposure on mosquito development has scarcely been addressed. There is dearth of information on the temporal temperature profiles of field breeding and development sites in general. Standard practice for all research that involves the use of laboratory-reared mosquitoes utilizes mosquitoes that are reared at a constant temperature. To date, the effect of exposure to diurnal temperature fluctuations during larval development has received little attention. In experiments looking at the role of DTR on disease transmission, mosquito vectors were reared under standard rearing conditions with no fluctuations in temperature. In fact, Paaijmans et al (52) showed that diurnal temperature variation in standard anopheline breeding sites resulted in faster development times than predicted based on mean ambient

temperature alone. One of the few studies examining the effect of diurnal temperature variation on mosquito development reported a highly female skewed sex ratio at higher temperatures and a lack of correlation between adult body size and rearing temperature (42). Larval exposure to diurnal temperature fluctuations in the field could well explain the results of Tun-Lin et al (66) who showed that correlations between wing length and temperature in field populations of *Ae. aegypti* were much lower than that for laboratory-reared mosquitoes. Carrington et al. (18) incriminated diurnal temperature fluctuations for this inconsistency when they examined the effect small (8°C) and large (18°C) diurnal temperature ranges had on select life-history traits in a strain of *Ae. aegypti* from Thailand. They showed that differing DTRs can have an effect on immature development time, survival to adulthood and female reproductive output.

INSECTICIDE RESISTANCE

Insecticide resistance, or a decrease in the degree of susceptibility to a given concentration of an insecticide, is generally considered to be an evolutionarily expensive trait that imposes fitness costs in a pesticide-free environment. Most commonly, resistance is thought to be solely the result of physiological and/or behavioral modifications that lead to lack of target site binding, increased detoxification activities, increased sequestration, or avoidance of contact with the chemical (16; 30; 60). However, relatively little has been reported on the actual costs imposed on a resistant phenotype (3). Insecticide resistance costs reported to date include reductions in pre-imaginal survival (9, 22), decreased fecundity (25), reduced longevity (3, 13), mating competition costs (10), and increased predation costs (11). Additionally, Rivero et al. (58) showed that, on average, resistant *Cx. pipiens* emerged as adults with 30% less

energetic reserves than susceptible mosquitoes. Interestingly, this cost was incurred during metamorphosis, as there was no difference in energetic reserves among resistant and susceptible fourth instar larvae. The quantity of energetic reserves accrued during larval development has also been suggested to influence several factors that influence vector competence and vectorial capacity (59).

The general consensus views insecticide resistance in terms of a trait that is expressed if possessed. However, it is possible that biotic and abiotic factors could influence the degree of susceptibility of mosquitoes to a specific insecticide. Some of these influences are relatively intuitive. For instance, larval development in the presence of adequate nutritional resources may allow developing larvae to allocate more resources to detoxification mechanisms, ultimately yielding adults better equipped to detoxify xenobiotics. Indeed, the "silver spoon" hypothesis posits that larvae developing in the presence of abundant nutritional resources produce healthier adults better able to cope with the vagaries of an unpredictable environment (24). In support of this theory Oliver and Brooke (49) showed that larval nutritional status can influence adult susceptibility to DDT in laboratory strains of *Anopheles arabiensis* Patton. Adult nutritional status (blood-fed, sugar-fed, or starved) has also been shown to influence behavioral responses to insecticide exposure (63).

Other parameters, such as water quality and population age structure, may also effect the expression of insecticide resistance. Tene Fossog et al. (64) suggested that water quality parameters influence larval susceptibility to pyrethrins but the influence of changing kdr allele frequencies in the source populations could not be ruled out. Several laboratory studies have shown a decrease in phenotypic resistance in older mosquitoes

(31, 56) and age since emergence of adult *An. gambiae* s.l. has been shown to influence susceptibility to deltamethrin, permethrin, malathion, DDT, and propoxur (19). In essence, Chouaibou et al (19) showed that older mosquitoes were more susceptible to the tested insecticides than younger mosquitoes.

Coustau et al (21) noted that, while some authors presented evidence of resistance associated fitness costs, others were unable to detect a cost. They suggested that resistance costs might only become apparent under a specific set of environmental conditions. Also, while the genetic determinants of insecticide resistance have been elucidated (14, 26, 69), the plasticity of phenotypic expression, to my knowledge, has never been examined. Bourguet et al. (14) did examine the plasticity of resistance allele dominance and found that the dominance of resistance expression (due to an insensitive acetylcholinesterase allele in *Cx. pipiens*) was dependent on the environmental variables examined: larval density, container depth, and daylight length. They showed that the recessivity of resistance was associated with more demanding environments. In other words, resistance heterozygotes expressed a resistant phenotype only under optimal rearing conditions. Given this, it is reasonable to assume that abiotic environmental variables could influence the cost and expression of insecticide resistance in a given population.

Insecticide Resistance and the Thermal Environment

The influence of ambient temperature on insecticide resistance has received little attention to date. Nayak and Collins (48) showed that treatment temperature accounted for 75% of the variability in time to population extinction for a phosphine resistant psocid; that is, temperature being more important than concentration of phosphine used.

Kikankie et al (34) evaluated the effect of ambient temperature on the use of an entomopathogenic fungus for control of resistant and susceptible *An. arabiensis*. While they focused on the viability of the fungal agent at different temperatures, their results showed that both resistant and susceptible strains of *An. arabiensis* exhibited significantly lower fungus induced mortality at lower ambient temperatures (21°C vs. 25°C). At a more physiological level, Yan et al (71) showed that a single-copy *Apis cerana* Fabricius glutathione S-transferase gene transcript, involved in oxidative stress protection, could be significantly up-regulated during exposure to thermal stress.

Research Goals

The focus of the present work was to evaluate the influence of diurnal temperature variation during larval development on selected adult traits in two prominent disease vectors, *Ae. aegypti* and *An. gambiae*. A variety of traits were assessed including adult lifespan, blood feeding and oviposition rates, fecundity, fertility and gonotrophic cycle length. In addition, adult susceptibility to three commonly used insecticides was assessed for both species to evaluate whether daily thermal variation during larval development could influence adult susceptibility to permethrin, malathion, and propoxur. Understanding the effects daily thermal variation during larval development has on epidemiologically important life history traits and insecticide susceptibility in adult mosquitoes is vital for accurate epidemiologic modeling and the development of effective disease mitigation strategies.

DTR effects on *Aedes aegypti* life history traits

Carrington et al. (18) was the first study to examine the effect small (8°C) and large (18°C) diurnal temperature fluctuations during larval development have on select

adult life-history traits. This is the only work that explores the influence exposure to diurnal thermal variation during larval development has on important adult life-history traits in a vector population. Their work showed that differing DTRs have an effect on immature development time, survival to adulthood, and female reproductive output. The first goal of this research was to expand upon the observations of Carrington et al. (18) by examining the influence of larval DTR exposure on adult blood-feeding frequency, fecundity, fertility, survival and developmental success rates, and sex ratio. The second goal was to determine whether the direction of any shift in measured traits was strain specific by utilizing two geographically isolated strains of *Ae. aegypti*, one strain from Thailand and one from Belize. Understanding the influence larval DTR exposure may have on epidemiologically important traits will aid in the development of more reliable estimates of disease threats.

DTR effects on *Aedes aegypti* insecticide susceptibility

Some authors have shown that short-term exposure of mosquito larvae to thermal stress can affect adult mosquito characteristics (43, 47). However, Raghavendra et al (55) is the only study that examined the role that larval thermal stress plays in adult susceptibility to an insecticide. They showed that larvae that were exposed to brief periods of thermal stress at 37°C and 39°C exhibited adult LT_{50} times to 5% malathion impregnated bed nets 1-3 fold longer than larvae reared at a constant 27°C. If brief exposure to thermal stress during larval development can influence adult susceptibility to insecticides, it is reasonable to expect that larval DTR exposure could influence adult insecticide susceptibility as well. In this study we assess the influence larval DTR exposure has on the adult susceptibility of two geographically distinct populations of *Ae.*

aegypti to three common insecticides; permethrin, malathion, and propoxur. Chemicals were selected to represent the two most common pathways targeted by chemical control. Each chemical has a distinct mode of action but malathion and propoxur target the same pathway. Permethrin targets ion channels in the nerve cell while malathion and propoxur target the aceytlcholinesterase system in the synaptic gaps between nerve cells. The main goal of this study was to determine whether larval DTR exposure could influence adult susceptibility and, if so, whether the direction and magnitude of this effect was consistent across geographically isolated populations. A secondary goal was to see whether any observed effects were non-selective, or if they differentially affected the two major systems targeted by conventional insecticides. Standard practice in resistance screening utilizes field caught mosquito populations reared through the F1 generation under standard laboratory conditions. Understanding the role larval DTR exposure plays in adult susceptibility to insecticides will help to define the limitations inherent in laboratory assays for insecticide resistance.

DTR effects on *Anopheles gambiae* life history traits

Insecticide resistance is generally considered to be an evolutionarily expensive trait that imposes fitness costs in a pesticide-free environment. Most commonly, resistance is thought to be solely the result of physiological and/or behavioral modifications that lead to lack of target site binding, increased detoxification activities, increased sequestration, or avoidance of contact with the chemical (16, 30, 60). Few studies have reported actual costs imposed on a resistant phenotype (3). Insecticide resistance costs reported to date include reductions in pre-imaginal survival (9, 22), decreased fecundity (25), reduced longevity (3, 13), mating competition costs (10), and

increased predation costs (11). The goal of this study was to determine whether the DTR during larval development imposed differential fitness costs between a susceptible and a knockdown resistant strain of *An. gambiae*. In this experiment, parameters of epidemiologic importance, such as blood-feeding frequency, fertility (estimated through egg viability rates), and survival rates for starved, glucose-fed and blood-fed cohorts were evaluated after exposure to several DTR regimens during larval development. Understanding the differential influence larval DTR exposure may have on epidemiologically important traits in resistant and susceptible vector populations will aid in the development of more reliable estimates of disease threats and mitigation strategies.

DTR effects on insecticide resistance in *Anopheles gambiae*

Resistance is often viewed in terms of a trait that is expressed if possessed. However, it is possible that biotic and abiotic factors could influence the degree of susceptibility of mosquitoes to a specific insecticide. Oliver and Brooke (49) showed that larval nutritional status can influence adult susceptibility to DDT in laboratory strains of *An. arabiensis*. Adult nutritional status (blood-fed, sugar-fed, or starved) has also been shown to influence behavioral responses to insecticide exposure (63). Tene Fossog et al. (64) suggested that water quality parameters can influence larval susceptibility to pyrethrins. In addition, several laboratory studies have shown a decrease in phenotypic resistance in older mosquitoes (31, 56). Furthermore, adult age in *An. gambiae* s,l. has been shown to influence susceptibility to deltamethrin, permethrin, malathion, DDT, and propoxur (19). Additionally, Bourguet et al (14) examined the plasticity of resistance allele dominance and found that dominance was inversely related to the severity of environmental factors.

The main goal of this study was to determine whether larval DTR exposure could influence adult susceptibility and/or resistance status and, if so, whether the direction and magnitude of this effect was consistent despite the presence of resistance alleles. To do this, two strains of *An. gambiae* were used, the G3 strain originally from The Gambia and the knockdown resistant AKRON strain from Benin. Also, the use of multiple insecticides allowed us to explore whether any observed effects from larval DTR exposure was general or mode of action specific. Understanding the influence larval DTR exposure has on insecticide resistance will help to define the limitations inherent in laboratory assays for insecticide resistance.

CHAPTER 2: Influence of Diurnal Temperature Fluctuations during Larval Development on Select Life History Traits in the Mosquito *Aedes aegypti* (Linnaeus)

ABSTRACT

Mosquito breeding sites are continually exposed to the vagaries of changing climactic conditions. This includes exposure to changing mean temperature caused by cyclical climactic shifts, such as the El-Nino Southern Oscillation, as well as short-term cyclical changes in ambient temperature caused by solar warming during the day and cooling at night. It can be argued that short-term daily fluctuations in temperature can influence mosquito life history traits much more drastically than shifts in long-term mean ambient temperature, whether over the course of weeks, months, or years. Long-term shifts only manifest in the life history traits of successive generations while diurnal exposure has the potential to influence life history traits of individuals within a single generation. Despite this, the majority of work in this area has focused on the role of static mean temperatures in estimating life history traits. This tendency to focus on mean temperature for defining the thermal environment extends to the use of epidemiological models for examining variables and predictive outcomes of vector-pathogen systems. If field exposure to fluctuating temperatures during larval development can influence life history trait expression then utilizing mean temperature as a parameter in any model may under or overestimate the degree of effect of that parameter on traits of interest. In this study, we examine the influence of diurnal temperature fluctuations during larval development on selected adult life history traits. Four cohorts of the dengue vector, *Aedes aegypti*, from two geographically isolated areas (Belize and Thailand) were

exposed as larvae to one of four diurnal temperature range (DTR) treatments from 0°C to 20°C around a daily mean of 28°C. Results suggest that larval exposure to diurnal temperature fluctuations influence the outcome of epidemiologically relevant life history traits and that these outcomes are strain/location specific.

INTRODUCTION

The thermal environment in which a poikilothermic organism finds itself has a major influence on the function of required physiological processes. For instance, warmer temperatures have been shown to increase hemolymph pH in the locust *Schistocerca nitens* (29) and hemolymph acidosis recovery rates in the grasshopper *Melanoplus bivittatus* (28). Also, ambient temperature is known to be an important constraint on the insect immune response (12) while small changes in ambient temperature have the ability to influence the outcome of insect-parasite interactions (65). Indeed, Benelli (8) showed that this influence can be carried over to the adult stage as larval exposure to low temperatures could reduce encapsulation rates in adults.

In addition, ambient temperature has been shown to influence measured life-history traits in vector populations (4, 39, 57) as well as alter disease transmission dynamics. For example, Westbrook et al. (70) showed that rearing *Ae. albopictus* at 18°C produced larger females that were six times more likely to be infected by Chikungunya virus (CHIKV) than when reared at 32°C. Additionally, Watts et al. (68) showed that, while *Ae. aegypti* could become infected with dengue virus (DEN-2) at any temperature from 20°C to 35°C, only those mosquitoes held at 30°C or higher were able to subsequently transmit the virus to monkeys, regardless of infectious dose. However, the influence ambient temperature has on vector/pathogen interactions is not consistent

across systems. For instance, higher temperatures increase infection rates of Rift Valley fever virus in *Cx. pipiens* (67) while decreasing infection rates of Eastern Equine Encephalitis virus in *Cx. tarsalis* (36).

Mosquito breeding sites are continually exposed to the vagaries of changing climactic conditions. This includes exposure to changing mean temperatures caused by cyclical climactic patterns, such as the el-nino southern oscillation, as well as short-term cyclical changes in ambient temperature caused by solar warming during the day and cooling at night. In terms of mosquito biology/physiology it can be argued that short-term daily fluctuations in temperature can influence mosquito life history traits much more dramatically than shifts in long-term mean ambient temperature, whether over the course of weeks, months, or years. These long-term shifts provide evolutionary forces a chance to shape the phenotype through continued fine-tuning of optimal life history traits across successive generations. However, exposure to daily cyclical changes also has the potential to influence the outcome of life history traits of individuals within a single generation. This could lead to differing outcomes for the same measured traits from intra-generational cohorts exposed to differing conditions (phenotypic plasticity), leading to greater observed variability of these traits in a natural population. As a result, natural populations may be better able to cope with long term shifts in mean temperature than may be presumed based on fitness estimates from lab reared cohorts. Long (40) suggested that overall population fitness tends to be greater in environments with more frequent fluctuations in temperature while Beardmore and Levine (5) showed that diurnal temperature fluctuations produce *Drosophila psuedoobscura* (Frolova) larvae with higher viability.

Despite this, the majority of disease vector work to date has focused on the role of static mean temperatures on pathogen-vector systems. However, daily temperature fluctuation, or the diurnal temperature range (DTR), has recently been the subject of some inquiry. Lambrechts et al. (38) showed that *Ae. aegypti* from Thailand were less susceptible to dengue virus infection and exhibited faster mortality at larger DTRs around the same mean ambient temperature. In doing so, they showed that the defining predictive factor for dengue virus transmission in Thailand might not be mean seasonal temperature or rainfall but the size of the DTR around the mean daily temperature. Additionally, Paaijmans et al. (53) showed that fluctuation in diurnal temperatures reduces the impact of increasing mean temperatures when using a thermodynamic model for malaria development (model parameters 18-28°C). Specifically, they showed that diurnal temperature fluctuations around means >21°C slow parasite development while fluctuations around means <21°C increases parasite development. Expanding on this model, Paaijmans et al. (50) showed that, at the extremes, diurnal temperature fluctuation facilitates transmission at lower mean temperatures and inhibits transmission at higher mean temperatures than that predicted by the standard static mean temperature models. As a result, current models for predicting malaria transmission risk may underestimate transmission at the fringes of endemic zones, such as in the Highlands of East Africa, while, at the same time, overestimating the risk in warmer portions of endemic zones (50).

While these investigations focused on the effect of DTR on adult mosquito parameters, field mosquito development sites are exposed to the same daily warming and cooling cycles that adult mosquitoes experience. Adult mosquitoes, however, have the

ability to moderate their exposure to daily temperature fluctuations through behavioral avoidance, e.g. responding to changes in microclimactic cues via the search and selection of more suitable resting locations. In contrast, exposure of mosquito larvae to diurnal temperature fluctuations is determined by the temporal characteristics of the development site. While these fluctuations may be minimal for species that breed in large bodies of water, species that utilize small ephemeral sites such as small puddles, tire ruts, hoof prints, discarded tires and water holding tanks experience greater instability—especially if these locations receive direct sunlight for any portion of the day. Given this, container breeders, such as *Ae. aegypti*—the predominant vector of dengue-dengue hemorrhagic fever—are routinely exposed to daily temperature fluctuations during larval development. This species is well known for its utilization of small containers for breeding. Many of these sites, such as discarded tires sitting in the sun, can amplify the natural ambient temperature fluctuations due to their low albedo. As a result, a large amount of incident solar radiation is absorbed leading to increases in day-time heating.

Given the exposure of some larval mosquito populations to diurnal changes in temperature, it is surprising that the effect of this exposure on mosquito development has scarcely been addressed. There is little information on the temporal temperature profiles of field breeding and development sites in general. Standard practice for all research that involves the use of laboratory-reared mosquitoes utilizes cohorts that are reared at a constant temperature. The effect of diurnal temperature fluctuations during larval development has all but been ignored. In prior experiments looking at the role of DTR on disease transmission, mosquito vectors were reared under standard rearing conditions with no fluctuations in temperature. In fact, Paaijmans et al. (52) showed that typical

anopheline larval habitats in the field exhibited diurnal temperature fluctuations with a net effect of maintaining mean habitat water temperatures 4-6°C higher than mean ambient temperatures. This difference resulted in faster development times than predicted based on mean ambient temperature alone. One of the few studies examining the effect of diurnal temperature fluctuations on the development of mosquito larvae documented a highly skewed sex ratio towards greater numbers of females at higher temperatures and a lack of correlation between adult body size and rearing temperature (42). Larval exposure to diurnal temperature fluctuations in the field could well explain the results of Tun-Lin et al. (66) who showed that correlations between wing length and temperature in field populations of *Ae. aegypti* were much lower than that for laboratory-reared mosquitoes.

Carrington et al. (18) suggested as much when they examined the effect small (8°C) and large (18°C) diurnal temperature fluctuations have on select life-history traits of a strain of *Ae. aegypti* from Thailand. This is the only work that explores the influence of larval exposure to diurnal temperature fluctuations on important adult life-history traits in a vector population. Their work showed that differing DTRs have an effect on immature development time, immature survival and female fecundity. Here, we examine the influence of larval DTR on adult blood-feeding frequency, fecundity, fertility (estimated through egg viability rates), survival rates for starved, glucose-fed and blood-fed cohorts, developmental success rates, and sex ratio. We also examine whether the influence of larval DTR exposure is strain/location dependent through paired experiments carried out on two geographically isolated strains of *Ae. aegypti*, one strain from Thailand (TH) and one from Belize (BZ).

METHODS

Mosquito Strains

Belize *Aedes aegypti* eggs originated from field collected mosquitoes collected in San Louis, Belize (18°11'38.6"N, 88°36'11.6"W) while Thailand *Ae. Aegypti* eggs were obtained from field collected specimens collected from Kanchaburi Province, Thailand. Eggs from both strains (F2) were sent to the labs at the Uniformed Services University of Health Sciences (USUHS) in Bethesda, MD where all experiments were conducted. Eggs were periodically reared through a generation under standard rearing conditions (28°C, LD 12:12, RH 80%) to maintain viable eggs for experimental use. All experiments were conducted using F3-F5 stock.

Rearing Conditions

Eggs were hatched in a vacuum chamber and larvae were allowed to develop undisturbed for 24 hours under standard rearing conditions (28°C, LD 12:12, RH 80%). One day old larvae were separated at a density of 50 individuals per 500ml cup containing 450ml dH_2O and reared according to treatment regimen. Each cup was fed a total of 0.25g of Cichlid Gold® large pellet fish food (Hikari, Kyorin co., ltd., Japan) over the course of larval development (0.05g ground on day 1 and one pellet (0.1g) on days 2 and 5). Water in each cup was refreshed on day 5 by decanting 350ml from each cup before adding 350ml fresh dH_2O pre-warmed to incubator temperature. Pupae were collected with disposable pipettes and placed in 500 ml emergence cups in 1 gallon plastic buckets. Pupae and adults were maintained under standard rearing conditions (28°C, LD 12:12, RH 80%) and provided 10% sucrose cotton pledglets ad libitum. All mosquitoes were reared using Percival I-36VL incubators (Percival Scientific, Perry IA).

Treatments

Larval cohorts were reared under 1 of 4 daily temperature treatments (LD 12:12, RH 80%) from 24hrs post hatch to pupation. Temperature treatments consisted of a DTR of 0°C, 10°C, 15°C and 20°C around a mean of 28°C. Daily temperature curves (except for the 0°C range) followed a truncated sine wave progression during the light phase (day) and a decaying exponential progression during the dark phase (night). These curves have been found to accurately mimic diurnal variation in soil and air temperature during the course of a solar day (54). One cohort from each strain was reared under each DTR treatment for a total of 8 cohorts. Both air and water temperature profiles were monitored with HOBO data loggers (Onset, Cape Cod, MA). Mean daily air temperature ranged from 27.4°C to 28.1°C across all treatments while mean daily water temperature ranged from 27.4°C to 27.8°C. All treatment daily mean temperatures were within 0.2°C of each other.

Developmental Success and Survival Evaluations

The following experiments were conducted to evaluate the effect of the four DTRs on immature development and adult longevity. For immature developmental success (percent pupation and percent emergence) and sex ratio, 200 one-day old mosquito larvae (4 cups of 50 each) were separated and reared according to the above treatment protocols. Pupae were counted and collected as above, date of pupation recorded, and allowed 5 days to emerge. At the end of the 5 days adult containers were placed in a -20°C freezer and left overnight. The following day the dead mosquitoes were sorted by sex and counted. For the creation of survival curves groups of 30 male and 30 female mosquitoes were separated for each cohort. Cohorts were set up 24hrs

post-emergence in 1 gallon plastic buckets with netted lids and held under standard rearing conditions (28°C, LD 12:12, RH 80%). Starved mosquito cohorts were given access to water soaked cotton pledglets ad libitum with mortality recorded daily while sucrose fed cohorts were given 10% sucrose pledglets ad libitum. Sugar-fed mosquitoes were given access to 10% sucrose solution via soaked cotton pledglets, ad libitum. Water and sucrose pledglets for the starved and sucrose survival analyses, respectively, were placed on the netted lids and refreshed daily. Mortality was recorded every 24 hours. Blood fed survival analyses were based on daily mortality data collected from cohort isolines described below.

Female Isolines

To estimate the influence DTR has on mosquito fecundity, gonotrophic cycle, blood feeding frequency, and percent egg hatch 30 female isolines were set up for each mosquito cohort. Buckets containing 200 female and 50 male three-day old adults were provided a bloodmeal and were allowed to rest/mate for 24 hours. Individual engorged females were then placed in 40-dram plastic collection vials (Bioquip inc., Ranch Dominguez, CA). Vial lids were modified by cutting out a centrally located circle 3cm in diameter and gluing untreated mosquito netting in place with a hot glue gun. Before placing a mosquito in each vial an oviposition funnel was made from 9 cm round filter paper (Fisher Scientific, Pittsburgh, PA) and placed at the bottom of the vial with a small amount of dH_2O. Isolines were maintained under standard rearing conditions (28°C, LD 12:12, RH 80%) and each female given the opportunity to blood feed for 10 minutes every Monday, Wednesday, Friday, and Saturday. Any mosquito still feeding at the end of the 10 minute interval was allowed to feed to repletion. Isolines were not provided

sucrose meals as some evidence suggest *Ae. aegypti* have a tendency to forego sugar meals in the field (27). Mosquitoes that laid eggs on non-blood feeding days (Tuesday, Thursday, and Sunday) were given a 10-minute opportunity to take a bloodmeal. After blood feeding, mosquitoes that laid eggs were transferred to a new 40-dram vial with a fresh oviposition funnel. Eggs were then counted and prepared for hatch. After counting, water was decanted from the vial and the filter paper containing eggs allowed to dry in an incubator (28°C, LD 12:12, RH 80%) for 48hrs. The filter paper and eggs were submerged in dH2O before placing the vial in a vacuum chamber for 20 minutes. Vials were then removed and placed in an incubator (28°C, LD 12:12, RH 80%) overnight. The number of hatched larvae was then counted the following day. The length of the gonotrophic cycle was measured as the number of days between the first blood meal and the first eggs laid. This first blood meal after egg deposition was considered the start of a new gonotrophic cycle. Interestingly, mosquitoes that had started, but not finished, laying a clutch of eggs when the next blood meal was offered refrained from feeding.

Statistics

All comparisons focused on analyzing differences between the 0°C cohort and all other cohorts for each strain. Developmental success rates were compared using binary logistic regression to obtain chi square scores for each comparison. Number of larvae that failed to pupate was found by subtracting the total number of pupae from the starting cohort number. Number of pupae that failed to emerge was found by subtracting the total number of adults that emerged from the total number of pupae for their respective cohorts. Survival analyses were compared using Kaplan-Meier methods with one time

unit equal to one day and case data weighed by the number of mosquitoes that died on each day. Female isoline data were compared using one-way ANOVA analyses for each species. Three variables were log-transformed (number of days between blood meals, gonotrophic cycle length, and number of blood meals per gonotrophic cycle) and one was square root transformed (hatch rate) to normalize the distribution for each variable. All other variables (number of blood meals, number of gonotrophic cycles, total number of eggs, number of eggs per day, number of eggs per gonotrophic cycle, and lifespan) had distributions that approached normal in their raw form.

RESULTS

Developmental Success

Diurnal temperature fluctuations significantly affected percent pupation rates in both the Thailand and Belize *Ae. aegypti* cohorts (Belize Wald χ^2= 33.246, DF= 3, $p<0.001$; Thailand Wald χ^2 = 56.05, DF= 3, $p<0.001$) but the direction of the effect differed by geographical strain (Figure 1). For BZ *Ae. aegypti* cohorts increases in DTR resulted in small increases in pupation rate (0°C= 89%; 10°C DTR= 95%, Wald χ^2 = 5.291 , DF= 1, p= 0.02; 15°C= 90%, Wald χ^2 = 0.234, DF= 1, p= 0.628) with the exception of the largest DTR, where there was a significant decrease in pupation rate (20°C= 76%, Wald χ^2 = 10.998, DF= 1, p= 0.001). In contrast, the TH cohorts showed significant decreases in pupation rates with increasing DTR (0°C= 92%; 10°C DTR= 82%, Wald χ^2 = 8.191, DF= 1, p= 0.004; 15°C= 60%, Wald χ^2 = 46.644, DF= 1, p< 0.001; 20°C= 70% Wald χ^2 = 27.434, DF= 1, $p<0.001$). Significant strain by treatment interaction was detected as well (Wald χ^2 = 32.805, DF= 3, p< 0.001). In contrast, adult emergence from collected pupae was generally not affected by larval DTR exposure

(Belize Wald χ^2 = 2.088, DF= 3, p= .554; Thailand Wald χ^2 = 13.129, DF= 3, p= 0.004). Percent emergence for all BZ cohorts was between 97-99% while that for the TH cohorts was 93-99%. Only the 10°C DTR TH cohort showed a deviation in adult emergence with an emergence rate of 93% (Wald χ^2 = 6.516, DF= 1, p= 0.01), contributing all of the significance to the model. Emergence rates for the rest of the TH cohorts were between 98-99%. There was no strain by treatment interaction detected for this variable (Wald χ^2 = 7.068, DF= 3, p= 0.07).

Diurnal temperature fluctuations did not appear to have an effect on the adult sex ratio of BZ cohorts with ratios ranging from 1:1 (female:male) in the 0°C control group to 1.2:1 in the 10°C and 20°C DTR treatment groups. TH cohorts, in general appeared to have a slightly male dominated sex ratio when compared to the BZ cohorts with more adult males than females in all but the 15°C DTR cohort. The sex ratio of TH cohorts ranged from 0.8:1 for the 0°C control group to 1.3:1 for the 15°C treatment group.

Survival Analyses

Mean sucrose-fed adult female survival times ranged from 33.6 to 42 days across treatments for the BZ strain (pooled cohorts mean ± SE = 34.7 ± 1.032), whereas that for the TH strain ranged from 24.9 to 36.8 days (pooled cohorts mean ± SE = 29.2 ± 0.807). Male mean survival time ranged from 28.2 to 53.9 days for BZ cohorts (pooled cohorts mean ± SE = 40.8 ± 1.3) and 43.6 to 59.9 days for TH cohorts (pooled cohorts mean ± SE = 53.3 ± 1.428) (Table 1). Comparing survival curves across treatments within each strain using log-rank tests showed that DTR did not have a significant effect on female survival time for either strain, with the exception of the TH 15°C cohort (χ^2= 13.877, DF= 3, p< 0.001). For males, however, all DTR treatment cohorts were significantly

different than their respective 0°C DTR control cohort for each strain with the exception of the TH 10°C DTR cohort (Figure 2). For BZ males, increases in DTR reduced survival times whereas, in the TH cohorts, increasing DTR lead to increased survival times. Blood-fed survival in the TH cohorts was not affected by increasing DTR. Means for these cohorts ranged from 20.1 to 23.4 days (pooled cohorts mean ± SE = 21.4 ± .863). The BZ cohorts, on the other hand, showed a significant decrease in blood-fed survival time for each DTR above the 0°C control (Figure 3). Means ranged from 22.5 to 25.9 compared to 38.2 for the 0°C control group. However, there was no trend with increasing DTR. Starved survival did not differ significantly between the BZ cohorts with the exception of the 10°C DTR female cohort, which exhibited a reduced survival rate compared to the control 0°C cohort (χ^2= 10.088, DF= 3, p= 0.001). For the TH strain both, the 15 and 20°C DTR female cohorts were significantly different from their 0°C control cohort (χ^2= 4.967, DF= 3, p= 0.026; χ^2= 8.567, DF= 3, p= 0.003 respectively), but with opposing trends. The 15°C cohort showed a decrease of approximately one day mean survival time (5.9 ± 0.246) while the 20°C cohort showed an increase of approximately one day (7.4 ± 0.143) from the 0°C DTR cohort mean (6.7 ± 0.15). Thailand male cohorts exhibited significantly reduced survival rates at both the 10 and 15°C DTR (χ^2= 12.854, DF= 3, p< 0.001; χ^2= 31.96, DF= 3, p< 0.001 respectively) compared to the 0°C control cohort (table 1).

Female Isolines

For the BZ strain, only the lifetime number of blood meals was unaffected by DTR with an overall average of 6.4 bloodmeals (SE= ±0.42) across all treatments. One-way ANOVA analysis revealed that DTR exposure had a significant impact on all other

life-history traits measured (Table 2). The effect of DTR on these traits was almost completely limited to DTR vs. no DTR comparisons. All post hoc multiple comparisons of these traits between the treatments alone (10°C, 15°C, and 20°C DTR) using Tukey HSD were not significant with the exception of hatch rate. For hatch rate the 15°C cohort exhibited significant differences compared to both the 10 and 20°C cohorts, as well as to the 0°C control. Outside of these comparisons, there were no other significant differences detected between the 0°C control and the 10°C and 20°C treatment cohorts for this variable. Exposure to diurnal temperature fluctuations during the larval stage resulted in an increase in the mean number of gonotrophic cycles from 3.3 days (SE= ± 0.554) in the 0°C cohort to 4.63 (SE= ± 0.692), 6.0 (SE= ± 0.659), and 5.83 (SE= ± 0.738) in the 10, 15 and 20°C cohorts, respectively. An increase in egg production was also detected in the 10°C and 15°C cohorts compared to the 0°C control cohort (Figure 4). The average gonotrophic cycle length decreased from 11.9 days (SE= ±2.879) in the 0°C cohort to 4.3 (SE= ± 0.403), 3.5 (SE= ± 0.26), and 3.3 (SE= ± 0.135) in the 10, 15 and 20°C cohorts, respectively. Both the number of days between blood meals, as well as the total number of blood meals taken per gonotrophic cycle, were reduced by exposure to any DTR during larval development. The 0°C cohort exhibited an average of 11.1 (SE= ± 1.636) days between blood meals while taking an average of 1.8 (SE= ± 0.424) blood meals per gonotrophic cycle. Mean number of days between blood meals and number of blood meals per gonotrophic cycle were as follows: 3.4 (SE= ± 0.246) and 1.5 (SE= ± 0.091) for the 10°C cohort, 3.7 (SE= ± 0.119) and 1.1 (SE= ±0.033) for the 15°C cohort, and 3.4 (SE= ± 0.661) and 1.2 (SE= ± 0.219).

For the TH strain, only blood feeding frequency, length of gonotrophic cycle, number of blood meals per gonotrophic cycle, number of eggs laid per day, clutch size per gonotrophic cycle, and percent hatch were significantly altered by DTR exposure during larval development (Table 2). Post hoc multiple comparisons using Tukey HSD showed that, in only one trait, was significance a result of between treatment comparisons alone. The number of blood meals taken per gonotrophic cycle was only significant between the 15°C (mean= 1.7± 0.155) and 20°C (mean= 1.3± 0.051) cohorts. However, no significant differences were detected in comparisons between the treatment cohorts and the control cohort for this trait. For the other four traits, almost all significance was attributed to the differences between the 15°C DTR cohort and the 0°C control cohort. Larval exposure to DTR in the Thailand cohorts caused a decrease in egg production per day and per gonotrophic cycle (Figure 4). However, the reduction in egg production per day was only significant for the 15°C cohort (mean= 14.4± 2.095 vs. 0°C cohort mean= 23.6± 2.937) while the mean number of eggs per gonotrophic cycle was significantly lower in both the 15°C and 20°C cohorts (15°C= 64.7± 7.324; 20°C= 74.4± 3.394; compared to 0°C= 93.8± 4.452). Gonotrophic cycle length was longer only in the 15°C cohort at 4.9± 0.727 compared to the 0°C cohort mean of 3.4± 0.209. All other cohort differences were not significant (10°C= 3.2± 0.197; 20°C= 3.1± 0.637). Mean hatch rate, however, was significantly reduced in all three treatment groups from 57.8% in the 0°C control cohort to 40.8%, 32.3%, and 35.9% in the 10, 15, and 20°C cohorts, respectively.

DISCUSSION

Results from this study clearly show that diurnal fluctuations in the thermal environment during larval development can influence characteristics in the adult

population. Interestingly, this influence appears to be specific to the geographic location as the direction of the effect was not always in the same direction between cohorts from Thailand and those from Belize. Increasing diurnal fluctuations in the larval environment led to increased pupation rates among Belize *Ae. aegypti* while causing a steady decrease in pupation rates among TH cohorts. The trend observed in the TH cohorts is consistent with Carrington et al. (18), who also noted that increasing DTR reduced pupation rates in experiments with *Ae. aegypti* colonies from Kamphaeng Phet, Thailand. However, they observed a slight increase in pupation in their small DTR (7.6°C) cohort and a decrease in their large DTR (18.6°C) cohort while the current study detected a steady decrease with increasing DTR in the TH cohorts before a slight positive shift in the 20°C cohort (though still much reduced from the 0°C control). The non-DTR control cohorts had similar pupation rates in both studies—92% in this study vs. ~91% in the Carrington et al. (18) work. Carrington et al. (18) had also speculated that colonies from different geographic locations may produce variable results but that the overall trend would remain the same. Interestingly, the current study suggests that, while on a finer geographic scale this may be correct, at larger scales, with more formidable barriers to gene flow, the direction of the trend cannot be assumed to be the same.

Despite the significant influence DTR had on pupation rates, adult emergence and sex-ratio was generally not affected by DTR, or geographic location, in this study. While the 10°C cohort for the TH strain exhibited a significant decrease in adult emergence, the fact that this decrease was not detected in any other DTR cohort across both strains suggests that the result may have been due to some unknown external factor. In both the BZ and TH strains no clear trend in sex ratio shift was observed. While Mohammed and

Chadee (42) reported a highly female skewed sex ratio resulting from larval exposure to diurnal temperature fluctuations, this was not detected here. In fact, all but one TH cohort (15°C DTR) had male skewed sex ratios while sex ratios for the BZ cohorts were not skewed in either direction.

Adult survival analyses also showed that larval DTR exposure influences adult longevity when all other variables are equal and that the direction of this influence is dependent on the geographic origin of the mosquito cohorts. Increases in DTR lead to reduced sucrose survival times in male BZ cohorts but increased sucrose survival times in male TH cohorts. For blood-fed survival, increases in DTR lead to decreases in survival times among BZ cohorts while having no effect on TH cohorts. Interestingly, this trend was not observed in both the female sucrose and starved survival analyses, suggesting that larval exposure to diurnal temperature fluctuations may differentially affect the ability of Belize populations of *Ae. aegypti* to cope with the physiological demands of egg production.

While blood-fed survival decreased with increasing DTR in the BZ cohorts, the total number of blood meals in each cohort remained the same. This suggests that blood feeding frequency increased with DTR exposure and this, in fact, was the case. For the TH cohorts, however, blood feeding frequency and the total number of blood meals taken were not influenced by larval DTR exposure. Also, in the BZ cohorts, increases in larval DTR exposure resulted in increases in the total number of gonotrophic cycles as well as increases in egg production while the length of the gonotrophic cycle decreased. In the TH cohorts the total number and length of gonotrophic cycles remained the same while egg production decreased with increasing DTR exposure. Carrington et al. (18) detected

no effect on clutch size or gonotrophic cycle length with DTR exposure and a decrease in the total number of gonotrophic cycles. This was not consistent with the results from both the BZ and the TH strain tested here. This may be due to the geographic origin of the cohorts (the origin of their stock was roughly 500 km from the stock source used for the Thailand cohorts in this study) or it may be due to differences in study design. The Carrington et al. (2013) study utilized 10 females per cohort over a 14 day observation period. In this study we followed ~30 females per cohort from day three post emergence until death. Given that our average clutch sizes were generally larger than those reported in Carrington et al. (18) as well, this suggests that the difference in reported trends may be due to source population variation in the underlying characteristics measured and not an artifact of study design.

While some differences were detected in the egg hatch rate between the TH and BZ cohorts, this study design limited the amount of biologically useful information that could be gleaned. Egg hatch rates were counted after a 24 hr hatch time and treated as a total hatch count. This did not take into account any potential effect larval exposure to DTR could have on delayed hatch rates among cohorts. Also, all egg clutches were given 48 hours to develop before hatching. If DTR has an effect on egg development rates, particularly, slowing the egg maturation process, this further compounds the reliability of the hatch rate counts collected in this study. Either one of these scenarios would have the effect of decreasing the hatch rate reported here. Future studies looking at this variable would do well to count hatching eggs over the course of several days and possibly under multiple drying cycles.

In general, it appears that larval exposure to DTRs of different magnitude only affected measured adult characteristics at the larger DTRs tested for the TH strain. In the majority of traits examined here only pupation rates in the TH cohorts showed a clear trend with increasing DTR. All other variables that displayed significant differences between the TH cohorts were mostly significant between the 0°C control/10°C cohorts and the 15°C/20°C cohorts. The BZ cohorts, on the other hand, exhibited significance between the 0°C control cohort and the 10°C/15°C/20°C cohorts in a majority of the variables that exhibited significant effects by DTR. Some of these differences between the BZ 0°C cohort and the BZ DTR treatment cohorts were quite stark. For example, the average gonotrophic cycle length in the 0°C cohort was 11.9 (SE= ± 2.879) days compared to an overall average of 5.6 (SE= ± 0.729) days for all DTR treatments combined. Egg laying behavior in the BZ strain appeared to be altered as well. Females in the 0°C control cohort would routinely only lay a few eggs per day, taking multiple days to complete laying a clutch of eggs. Females in the 0°C TH cohort did not exhibit this behavior at all and usually deposited all eggs within 48 hours from the first eggs laid. This behavior was not evident in any of the treatment cohorts, and seemed to diminish with age in the BZ control cohort. Further investigation is needed to determine whether BZ *Ae. aegypti* approach egg deposition differently when reared at constant temperatures as opposed to a DTR regimen.

It is apparent that larval DTR exposure can alter the outcome of epidemiologically important traits and that the magnitude and direction of these effects appear to be specific to the population considered. Given this, several variables with the potential to influence vectorial capacity could be affected by DTR exposure, thus altering the estimate of a

populations' ability to propagate a pathogen of interest. Common variables that have the potential to be affected by temperature are, blood feeding frequency, survival, population density, and vector competence. While the conventional approach is to assume vector competence and vectorial capacity is temperature independent, Paaijmans et al. (51) has shown that this assumption leads to erroneous vectorial capacity estimates by temperature which, in the case of malaria transmission, overestimates transmission probability at warmer temperatures. Since vectorial capacity estimates concern themselves with adult mosquito populations, the potential influence of temperature on feeding frequency, survival and population density are fairly intuitive. However, less obvious is the potential effect the larval thermal environment may have on these variables. Results of this study indicate that larval exposure to DTR in *Ae. aegypti* can decrease pupation rates in the TZ strain while increasing them for the BZ strain. Additionally, egg production in the TH cohorts decreased with increasing DTR while that for the BZ cohorts increased. A net result, these two variables working in tandem could lead to a decrease in population density with increasing DTR for the Thailand population but an increase in population density for the Belize population. In effect, DTR could significantly alter the population density of a vector species even though the mean temperature remained the same.

The survival rate of the population can affect the population density as well. Larval exposure to DTR in the BZ strain resulted in significant reductions in blood fed survival. So, while egg production and pupation rates increased with DTR exposure, survival of reproducing (and, thus, blood feeding) adults decreased. This interplay could reduce the influence DTR has on adult population density for the BZ strain. This decrease in survival among blood feeding females was not seen in the TH cohort,

suggesting that DTR may have more of an effect on population density for this strain. In addition, the direction of the potential effect was strain specific. For the BZ strain DTR had the potential to increase population density while, for the TH strain, it would reduce it. Since it has been shown that *Ae. aegypti* is less susceptible to infection with dengue virus when exposed to large DTRs (38), DTR exposure could lead to higher population densities in the TH strain and lower population densities in the BZ strain during conditions that are optimal for virus infection of the mosquito. As a result, you could have the same vector/virus/environmental interactions but different infection rates in the human population.

This study highlights the need for a more robust understanding of how the thermal environment during larval development influences the expression of epidemiologically important traits in the adult population. Indeed, we suggest that expression of these traits may be far more plastic than previously thought, as this study shows that the direction of the effect is highly dependent on the population being examined. Given this, it is critical to evaluate variables for a given population beyond a single point in time and to examine the effect of changes in trait variability across time. Further studies are needed to elucidate the extent to which this plasticity is altered through repeated selective pressures under a specific thermal environment. Further, it is possible that the state of the thermal environment during larval development could influence vector competence and vectorial capacity in the adult population. Recent work has shown this to be the case (2, 43, 46, 47), however, these efforts have focused on differences between larvae reared at different mean temperatures or exposed to short-term thermal stressors. Studies are needed to determine what, if any, effect larval DTR exposure has on adult vector competence and

vectorial capacity. These studies should not only focus on species specific effects but intraspecific effects between different geographic strains as well.

Table 1. Mean survival times ± standard errors (in days) with log-rank test results for pairwise comparisons of sucrose, blood-fed, and starved survival curves constructed using Kaplan-Meier methods. Tested DTRs were compared against the control 0°C DTR cohort for each strain. Sample size for each DTR cohort ranged from 30 to 35 mosquitoes each. For all pairwise comparisons DF=3. * = significant at the p= 0.05 level.

Treatment		Belize	Thailand
Sucrose-fed			
Female	**0°C**	33.6±1.9	27.59±1.46
	10°C	30.88±0.81, χ^2= 3.005, p= 0.083	26.23±1.16, χ^2= 0.441, p= 0.507
	15°C	42.03±2.91, χ^2= 3.213, p= 0.073	36.78±1.5, χ^2= 13.877, p< 0.001*
	20°C	32.55±1.68, χ^2= 0.521, p= 0.47	24.94±1.37, χ^2= 2.27, p= 0.132
Male	**0°C**	53.93±2.68	43.56±2.28
	10°C	28.19±1.17, χ^2= 50.586, p< 0.001*	51.03±1.77, χ^2= 2.816, p= 0.093
	15°C	41.49±1.99, χ^2= 11.831, p= 0.001*	59.89±3.33, χ^2= 18.218, p< 0.001*
	20°C	41.12±2.33, χ^2= 12.586, p< 0.001*	59.85±2.99, χ^2= 25.297, p< 0.001*
Blood-fed			
Female	**0°C**	48.35±2.98	24.47±1.38
	10°C	26.72±2.79, χ^2= 11.031, p= 0.001*	24.07±1.49, χ^2= 0.029, p= 0.865
	15°C	29.88±2.59, χ^2= 7.426, p= 0.006*	27.44±1.98, χ^2= 2.16, p= 0.142
	20°C	29.0±2.79, χ^2= 9.384, p= 0.002*	26.04±2.1, χ^2= 1.062, p= 0.303
Starved			
Female	**0°C**	7.4±0.15	6.72±0.15
	10°C	6.55±0.17, χ^2= 10.088, p= 0.001*	6.2±0.27, χ^2= 0.024, p= 0.877
	15°C	7.31±0.14, χ^2= 0.148, p= 0.700	5.94±0.25, χ^2= 4.967, p= 0.026*
	20°C	7.00±0.18, χ^2= 2.257, p= 0.133	7.4±0.14, χ^2= 8.567, p= 0.003*
Male	**0°C**	8.19±0.16	9.07±0.17
	10°C	7.97±0.18, χ^2= 0.83, p= 0.362	7.97±0.22, χ^2= 12.854, p< 0.001*
	15°C	8.26±0.13, χ^2= 0.007, p= 0.933	6.72±0.38, χ^2= 31.96, p< 0.001*
	20°C	8.61±0.15, χ^2= 3.186, p= 0.074	8.91±0.23, χ^2= 0.155, p= 0.694

Table 2. Results of one way ANOVA analyses comparing selected life-history traits between female isolines from mosquito cohorts reared at a constant 28°C to those reared under diurnal temperature fluctuations of 10, 15, and 20°C. Sample size for each cohort consisted of 30 female mosquitoes each. * = significant at the p= 0.05 level.

Strain	Trait	F Statistic	P Value
Belize	Total # of blood meals	0.839, DF=3	P= 0.475
	Blood feeding frequency	45.054, DF=3	P< 0.001*
	Total # of gonotrophic cycles	3.472, DF=3	P= 0.018*
	Gonotrophic cycle length	16.915, DF=3	P< 0.001*
	# blood meals per gono. cycle	4.807, DF=3	P= 0.003*
	Total egg production	3.914, DF=3	P= 0.011*
	Eggs per day	9.659, DF=3	P< 0.001*
	Eggs per Gonotrophic cycle	3.704, DF=3	P= 0.014*
	Egg hatch rate	6.562, DF=3	P< 0.001*
	Lifespan	12.947, DF=3	P< 0.001*
Thailand	Total # of blood meals	0.103, DF=3	P= 0.958
	Blood feeding frequency	2.653, DF=3	P= 0.052
	Total # of gonotrophic cycles	0.132, DF=3	P= 0.941
	Gonotrophic cycle length	4.862, DF=3	P= 0.003*
	# blood meals per gono. cycle	2.817, DF=3	P= 0.042*
	Total egg production	1.476, DF=3	P= 0.225
	Eggs per day	3.929, DF=3	P= 0.01*
	Eggs per Gonotrophic cycle	6.672, DF=3	P< 0.001*
	Egg hatch rate	8.448, DF=3	P< 0.001*
	Lifespan	0.785, DF=3	P= 0.505

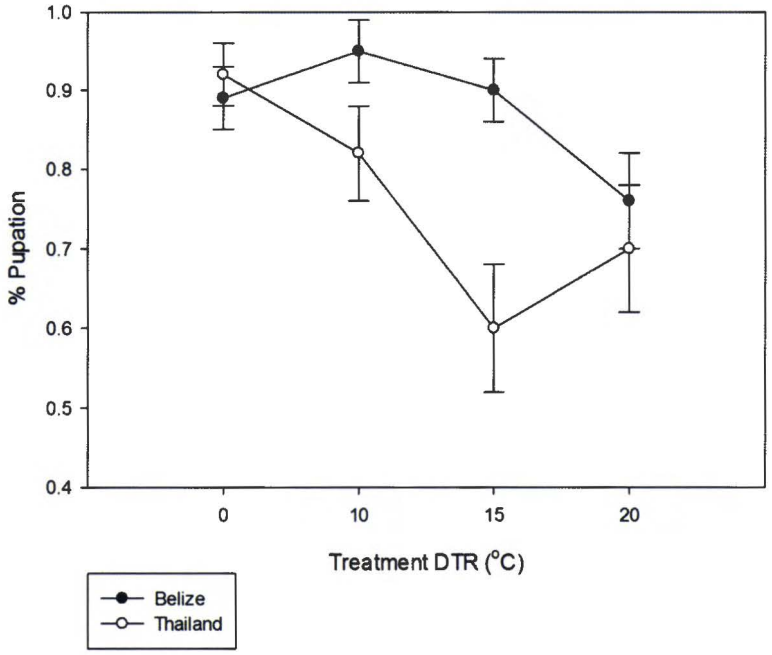
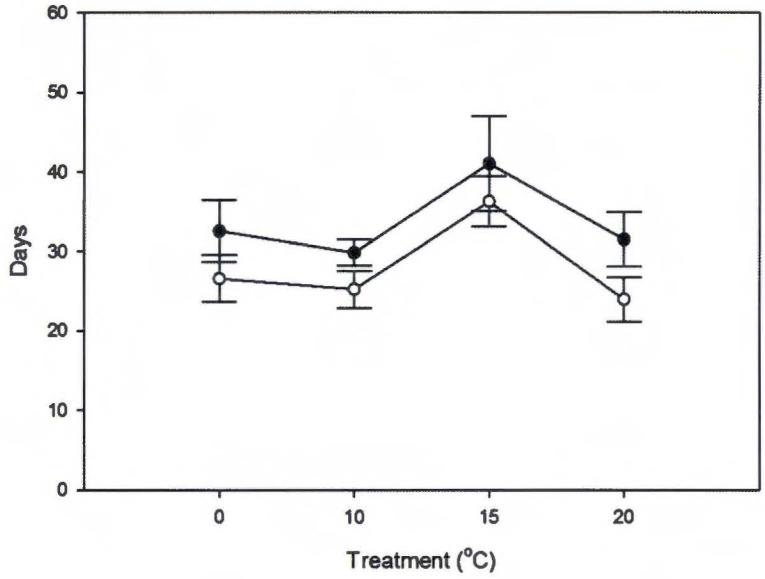


Figure 1. Mean pupation rates for each DTR cohort for both the Belize and Thailand strains of *Aedes aegypti*. Bars represent 95% confidence intervals.

a.



b.

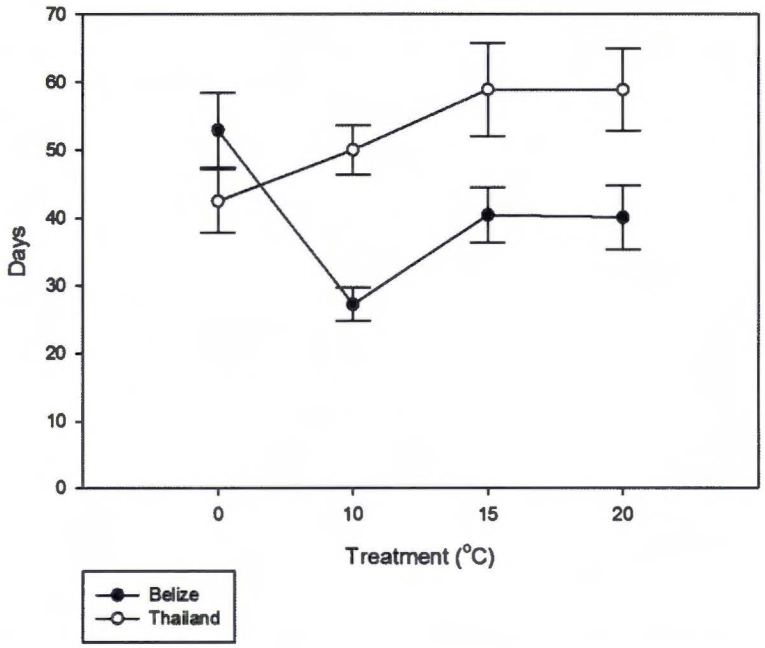


Figure 2. Mean female (a) and male (b) sucrose survival times in days post emergence for the Belize and Thailand strains of *Ae. aegypti* for each DTR treatment. Bars represent 95% confidence intervals.

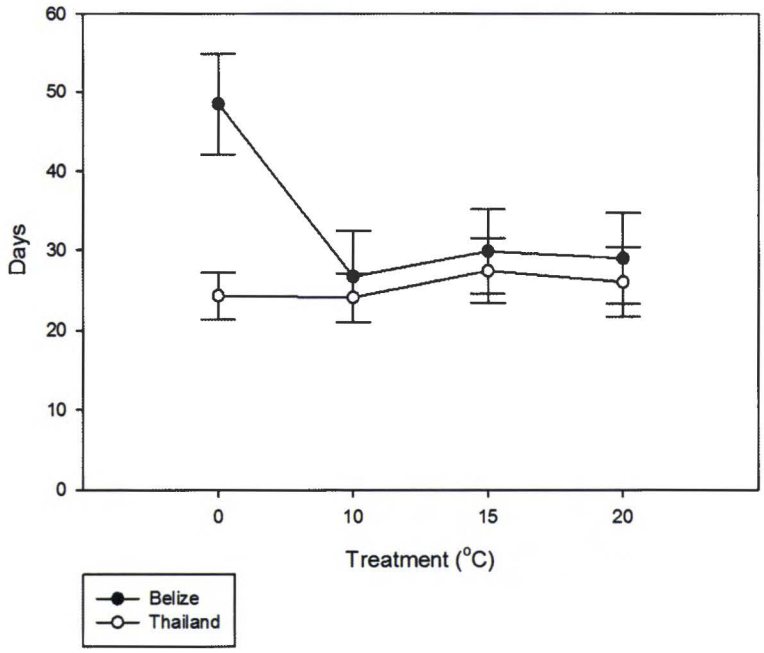


Figure 3. Mean bloodfed survival times in days post emergence for the Belize and Thailand strains of *Ae. aegypti* for each DTR treatment. Bars represent 95% confidence intervals.

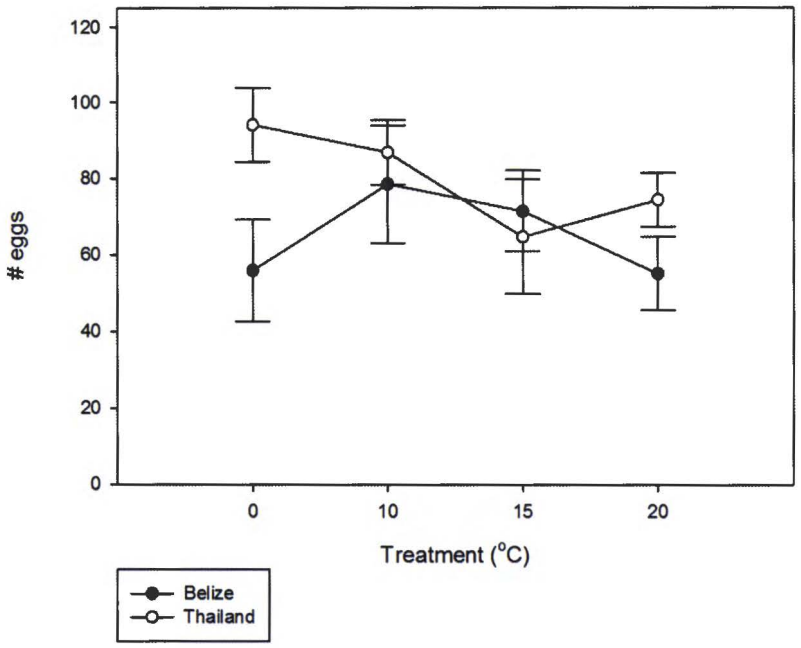


Figure 4. Mean egg clutch size for the Belize and Thailand strains of *Ae. aegypti* for each DTR treatment. Bars represent 95% confidence intervals.

CHAPTER 3: Larval DTR Exposure and Adult Susceptibility to Commonly Used Insecticides in the Mosquito *Aedes aegypti*

ABSTRACT

Larval development sites for container breeding mosquitoes are often exposed to daily fluctuations in ambient temperature, potentially affecting the thermal environment of developing larvae. While some work has been done focusing on the influence of the larval thermal environment on adult life history traits, no work to date has focused on what influence, if any, diurnal temperature fluctuations during larval development have on adult insecticidal susceptibility. Here we report that larval exposure to diurnal temperature fluctuations can influence adult susceptibility to insecticides in two strains of the dengue vector, *Aedes aegypti*, and that these effects are strain and pesticide specific. Increasing DTR exposure resulted in up to a four-fold increase in LD_{95} concentrations for a Thailand strain but small, insignificant differences, for a Belize strain of *Ae. aegypti*. However, for both propoxur and malathion, a small increase in DTR to 10°C from 0°C resulted in a small decrease in susceptibility for both the Belize and the Thailand strains. Despite the effects of larval DTR exposure on adult insecticide susceptibility, changes in susceptibility were not enough to have potential effects on the efficacy of field application rates for all three of the compounds tested.

INTRODUCTION

Ever since the World Health Organization (WHO) initiated the first malaria eradication campaign in 1955, the use of insecticides has been a cornerstone of vector management strategies. Since that time, the development of resistance to several

classes of insecticides has been an ongoing issue (16, 30, 33). Resistance, or a decrease in the degree of susceptibility to a given concentration of an insecticide, is influenced by multiple factors. Most commonly, resistance is thought to be the result of physiological and/or behavioral modifications that lead to a lack of target site binding, increased detoxification, increased sequestration, or avoidance of contact with the chemical (16, 30, 60). Also, it is generally accepted that the presence of insecticide resistance imposes fitness costs in a pesticide-free environment. Despite this, relatively little has been reported on the actual costs imposed on a resistant phenotype (3). Insecticide resistance costs currently recognized include reductions in pre-imaginal survival (9, 22), decreased fecundity (25), reduced longevity (3, 13), mating competition costs (10), and increased predation costs (11). Additionally, Rivero et al (58) showed that, on average, resistant *Cx. pipiens* emerged as adults with 30% less energetic reserves than susceptible mosquitoes. Interestingly, this cost was incurred during metamorphosis, as there was no difference in energetic reserves among resistant and susceptible fourth instar larvae. The amount of energy reserves accrued during larval development has also been suggested to influence several factors that determine vector competence and vectorial capacity (59).

Coustau et al. (21) noted that, while some authors presented evidence of resistance associated fitness costs, others were unable to detect a cost. They suggested that resistance costs might only become apparent under a specific set of environmental conditions. Also, while the genetic determinants of insecticide resistance are known (14, 26, 69), little has been done to examine the plasticity of phenotypic expression. Bourguet et al. (14) did study the plasticity of resistance allele dominance and found that expression of resistance (due to an insensitive acetylcholinesterase allele in *Cx. pipiens*)

was dependent on the environmental variables examined: larval density, container depth, and daylight length. They showed that the expression of resistance was associated with more demanding environments. In other words, heterozygotes expressed a resistant phenotype only under optimal rearing conditions. Given this, it is reasonable to assume that abiotic environmental variables could influence the cost and expression of insecticide resistance in a population.

The general consensus views insecticide resistance in terms of a trait that is both possessed and expressed or not. However, it is possible that biotic and abiotic factors could influence the degree of susceptibility of mosquitoes to a specific insecticide. Some of these influences are relatively intuitive. For instance, larval development in the presence of adequate nutritional resources may allow developing larvae to allocate more resources to detoxification mechanisms, ultimately yielding adults better equipped to detoxify xenobiotics. The "silver spoon" hypothesis posits that larvae developing in the presence of abundant nutritional resources produce healthier adults better able to cope with the vagaries of an unpredictable environment (24). In support of this theory Oliver and Brooke (49) showed that larval nutritional status can influence adult susceptibility to DDT in laboratory strains of *An. arabiensis* Patton. Adult nutritional status (blood-fed, sugar-fed, or starved) has also been shown to influence behavioral responses to insecticide exposure (63).

Other parameters, such as water quality and population age structure, may also have an effect on the expression of insecticide resistance. Tene Fossog et al. (64) suggested that water quality parameters influence larval susceptibility to pyrethrins but the influence of changing kdr allele frequencies in the source populations could not be

ruled out. Several laboratory studies have shown a decrease in phenotypic resistance in older mosquitoes (31, 56) and age since emergence of adult *An. gambiae* sl has been shown to influence susceptibility to deltamethrin, permethrin, malathion, DDT, and propoxur (19). In essence, Chouaibou et al. (19) showed that older mosquitoes were more susceptible to the tested insecticides than younger mosquitoes.

Ambient temperature might also have the ability to affect susceptibility to insecticide exposure considering that it has been shown to influence life-history traits of vector populations in general (4, 39, 57). Ambient temperature can also have a major influence on vector competence through alteration of interaction dynamics between pathogen and host. For example, Westbrook et al. (70) showed that rearing *Ae. albopictus* at 18°C produced larger females that were six times more likely to be infected by Chikungunya virus than when reared at 32°C. Additionally, Watts et al. (68) showed that, while *Ae. aegypti* could become infected with dengue virus (DEN-2) at any temperature from 20°C to 35°C, only those mosquitoes held at 30°C or higher were able to subsequently transmit the virus to monkeys, regardless of infectious dose. However, the influence ambient temperature has on vector/pathogen interactions is not consistent across systems. For instance, higher temperatures increase infection rates of Rift Valley fever virus in *Cx. pipiens* (67) while decreasing infection rates of Eastern Equine Encephalitis virus in *Cx. tarsalis* (36).

The influence of ambient temperatures during larval development on insecticide resistance is scarcely considered. Nayak and Collins (48) showed that treatment temperature accounted for 75% of the variability in time to population extinction for a phosphine resistant psocid; that is, temperature being more important than concentration

of phosphine used. Kikankie et al. (34) evaluated the effect of ambient temperature on the use of an entomopathogenic fungus for control of resistant and susceptible *An. arabiensis*. While they focused on the viability of the fungal agent at different temperatures, their results showed that both resistant and susceptible strains of *An. arabiensis* exhibited significantly lower fungus induced mortality at lower ambient temperatures (21°C vs. 25°C). At a more physiological level, Yan et al. (71) showed that a single-copy *Apis cerana* Fabricius glutathione S-transferase gene transcript, involved in oxidative stress protection, could be significantly up-regulated during exposure to thermal stress.

Perhaps a more important consideration than ambient temperature alone is that of daily temperature fluctuations, or diurnal temperature range (DTR). The influence of DTR on pathogen-transmission systems has only recently been the subject of attention. Lambrechts et al. (38) showed that *Ae. aegypti* from Thailand were less susceptible to dengue virus infection and exhibited faster mortality at larger DTRs around the same mean ambient temperature. In doing so, they showed that the defining predictive factor for dengue virus transmission in Thailand might not be mean seasonal temperature, or rainfall, but the size of the DTR around the mean daily temperature. Additionally, Paaijmans et al. (53) showed that diurnal fluctuations in temperature reduce the impact of increasing mean temperatures when using a thermodynamic model for malaria development (model parameters of 18-28°C). Specifically, they showed that diurnal temperature fluctuations around means >21°C slow parasite development while fluctuations around means <21°C increase parasite development. Expanding on this model, Paaijmans et al. (50) demonstrated that, at the model extremes, diurnal

temperature fluctuations make transmission possible at lower mean temperatures and reduces transmission at higher mean temperatures more than that predicted by the standard mean temperature models. As a result, current models for predicting malaria transmission risk may underestimate transmission at the fringes of endemic zones, such as in the Highlands of East Africa, while, at the same time, overestimating the risk in warmer portions of endemic zones (50). Despite the increasing focus given to the influence of DTR on adult vector-pathogen transmission dynamics, the influence of DTR on adult insecticide susceptibility has not been examined.

Just as adult mosquitoes are potentially exposed to diurnal temperature fluctuations in the field, the potential exists for larval development sites to be exposed to the same daily warming and cooling cycles. As such, the thermal environment of the developing larvae should be taken into consideration. The larval thermal environment, however, may be more important than that for adult mosquitoes because adults have the ability to moderate their exposure to daily temperature fluctuations through behavioral avoidance (e.g. responding to changes in microclimactic cues via the search and selection of more suitable resting locations). In contrast, exposure of mosquito larvae to diurnal temperature fluctuations is determined by the temporal characteristics of the development site. While these fluctuations may be minimal for species that breed in large bodies of water, species that utilize small ephemeral sites such as small puddles, tire ruts, hoof prints, discarded tires and water holding tanks are potentially much more exposed—especially if these locations receive direct sunlight for any portion of the day. Several prominent disease vectors routinely utilize such habitats as breeding sites. *Ae. aegypti*, a major vector of dengue-dengue hemorrhagic fever and a container breeder, is routinely

exposed to diurnal changes in temperature. This species is well known for its utilization of small containers for breeding and immature development sites. Many of these sites, such as discarded tires sitting in the sun, can amplify the magnitude of natural ambient temperature fluctuations due to their low albedo. As a result, a large amount of incident solar radiation is absorbed leading to greater increases in the temperature of the aquatic medium that supports larval development.

Given the exposure of some larval mosquito populations to diurnal changes in temperature, it is surprising that the effect of this exposure on mosquito development has scarcely been addressed. Indeed, there is dearth of information on the temporal temperature profiles of field breeding and development sites in general. Standard practice for all research that involves the use of laboratory-reared mosquitoes utilizes mosquitoes that are reared at a constant temperature. To date, the effect of exposure to diurnal temperature fluctuations during larval development has all but been ignored. In the prior experiments looking at the role of DTR on disease transmission, mosquito vectors were reared under standard rearing conditions with no fluctuations in temperature. While standardized, this regimen has little validity when compared to field development sites. This fact was illustrated by Paaijmans et al. (52) who showed that natural anopheline habitats in the field exhibited diurnal temperature fluctuations with a net effect of maintaining mean habitat water temperatures 4-6^{o}C higher than mean ambient temperatures. This difference resulted in faster development times than predicted based on mean ambient temperature alone. One of the few studies to date examining the effect of diurnal temperature fluctuations on the development of mosquito larvae showed a highly female skewed sex ratio at higher temperatures and a lack of

correlation between adult body size and rearing temperature (42). Results of experiments to be presented elsewhere showed that larval exposure to DTR can have multiple, conflicting, effects on life-history traits depending on the geographic origin of the population (Chapter 2). Indeed, larval exposure to diurnal temperature fluctuations in the field could well explain the results of Tun-Lin et al. (66) who showed that correlations between wing length and temperature in field populations of *Ae. aegypti* were much lower than that for laboratory-reared mosquitoes.

While not focused on diurnal temperature fluctuations specifically, some authors have shown that short-term exposure of mosquito larvae to thermal stress can affect adult mosquito characteristics. Muturi et al. (47) showed that *Ae. aegypti* larvae reared at 32°C were much more susceptible to infection with Sinbis virus as adults. They also showed that exposure to elevated temperatures during immature development led to a decreased expression of heat shock protein 83 and increased expression of defensin and cecropin in subsequent adults. The authors suggested that this led to gut fauna changes making the mosquitoes more susceptible to infection. Mourya et al. (43) had come to a similar conclusion when they examined the influence thermal stress on *Ae. aegypti* larvae had on adult susceptibility to CHIKV. The only work that examines the role larval thermal stress plays in the susceptibility of adults to an insecticide is that of Raghavendra et al. (55) who showed that larval exposure to brief periods of thermal stress resulted in one to three fold increases in adult LT_{50} times to 5% malathion impregnated bed nets.

If brief exposure to thermal stress during the larval stage can influence adult susceptibility to insecticides, it is reasonable to expect that DTR exposure could influence susceptibility as well. Standard practices used in resistance screening utilize field caught

mosquito populations reared through the F1 generation under standard laboratory conditions. Understanding the role larval DTR exposure plays in adult susceptibility will help to define the limitations inherent in laboratory assays for insecticide resistance as well as other evaluations that would benefit from further characterization of the laboratory test populations. In this study, we assess the influence larval DTR exposure has on the susceptibility of the BZ and TH strains of *Ae. aegypti* to three common insecticides; permethrin, malathion, and propoxur. Chemicals were selected to represent the two most common pathways targeted by chemical control. Each chemical has a distinct mode of action but malathion and propoxur target the same pathway. Permethrin targets ion channels in the nerve cell while malathion and propoxur target the aceytlcholinesterase system in the synaptic gaps between nerve cells. The main goal of this study was to determine whether larval DTR exposure could influence adult susceptibility and, if so, whether the direction and magnitude of this effect was consistent across geographically isolated populations. Also, the use of multiple insecticides allows us to test whether any observed effects are non-selective, or if they differentially affect the two major systems targeted by conventional insecticides.

METHODS

Mosquito Strains

Belize *Ae. aegypti* eggs originated from field collected mosquitoes collected in San Louis, Belize (18°11'38.6"N, 88°36'11.6"W) while Thailand *Ae. aegypti* eggs were obtained from field collected specimens collected from Kanchaburi Province, Thailand. Eggs from both strains (F2) were sent to the labs at the Uniformed Services University of Health Sciences (USUHS) in Bethesda, MD where all experiments were conducted.

Eggs were periodically reared through a generation under standard rearing conditions (28°C, LD 12:12, RH 80%) to maintain viable eggs for experimental use. All experiments were conducted using F3-F5 stock.

Rearing Conditions

Eggs were hatched in a vacuum chamber and larvae were allowed to develop undisturbed for 24 hrs under standard rearing conditions (28°C, LD 12:12, RH 80%). One day old larvae were separated at a density of 50 individuals per 500ml cup containing 450ml dH_2O and reared according to treatment regimen. Each cup was fed a total of 0.25g of Cichlid Gold® large pellet fish food (Hikari, Kyorin co., ltd., Japan) over the course of larval development (0.05g ground on day 1 and one pellet (0.1g) on days 2 and 5). Water in each cup was refreshed on day 5 by decanting 350ml from each cup before adding 350ml fresh dH_2O pre-warmed to incubator temperature. Pupae were collected with disposable pipettes and placed in 500 ml emergence cups in 1 gallon plastic buckets. Pupae and adults were maintained under standard rearing conditions (28°C, LD 12:12, RH 80%) and provided 10% sucrose cotton pledglets ad libitum. All mosquitoes were reared using Percival I-36VL incubators (Percival Scientific, Perry IA).

DTR Treatments

Larval cohorts were reared under 1 of 4 daily temperature treatments from 24hrs post-hatch through to pupation (LD 12:12, RH 80%). Temperature treatments consisted of a DTR of 0°C, 10°C, 15°C and 20°C around a mean of 28°C. Daily temperature curves (except for the 0°C range) followed a truncated sine wave progression during the light phase (day) and a decaying exponential progression during the dark phase (night). These curves have been found to accurately mimic diurnal variation in soil and air temperature

during the course of a solar day (54). One cohort from each strain was reared under each DTR treatment for a total of 8 cohorts. Both air and water temperature profiles were monitored with HOBO data loggers (Onset, Cape Cod, MA). Mean daily air temperature ranged from 27.4°C to 28.1°C across all treatments while mean daily water temperature ranged from 27.4°C to 27.8°C. All treatment daily mean temperatures were within 0.2°C of each other.

Insecticide Assays

To evaluate the influence larval DTR exposure has on adult mosquito susceptibility to selected insecticides a series of CDC bottle assays were conducted using permethrin, malathion, and propoxur. All insecticide assays were performed using the current MR4 CDC bottle assay protocol (17, 20). Briefly, 15-20 nulliparous sugar-fed 3-7 day old female mosquitoes were separated into holding tubes just before assay start. Assays consisted of five concentrations per chemical. Dosages used were originally calculated in nmol/cm^2 and selected to provide percent mortality counts with enough granularity to allow construction of probit transformed regression lines. All concentrations were then back-transformed to ng/cm^2 to allow easy comparison to standard field application rates. Insecticides were dissolved and diluted in molecular grade acetone and control bottles treated with acetone. Concentrations selected for use with Belize assays were 798.21, 199.55, 50.08, 12.52, and 3.13 ng/cm^2 for permethrin, 37.66, 18.83, 9.42, 4.6, and 2.3 ng/cm^2 for propoxur, and 21.14, 17.84, 14.54, 11.23, and 7.93 ng/cm^2 for malathion. Concentrations for the Thailand strain were 199.55, 50.08, 12.52, 3.13, and 0.78 ng/cm^2 for permethrin, 18.83, 9.42, 4.6, 2.3, and 1.15 ng/cm^2 for propoxur, and 49.55, 16.52, 11.56, 8.26, and 3.3 ng/cm^2 for malathion. Glass bottles

(250ml Wheaton) were treated with 1ml of solution, capped and swirled for 5 seconds to ensure treatment of the lid, Bottles were then placed on a bottle roller (Wheaton Science Products, Millville, NJ), with lid removed, and allowed to dry while rolling (approximately 5 minutes each). Bottles were then placed under a fume hood with lids off and loosely covered with aluminum foil overnight to allow for all acetone vapors to dissipate prior to testing.

Three pools of 15-20 mosquitoes were used for testing at each concentration and for each control. All assays were run for 60 minutes with percent knockdown being recorded at 15-minute intervals. Knockdown was defined as any mosquito unable to fly, walk, or rest in a deliberate manner. All mosquito pools were placed in one pint holding cages after 60 minutes, provided with a 10% sucrose pledglet and held at 28°C, 80% RH, LD: 12/12 for 24 hrs to obtain 24hr mortality counts. When control mortality exceeded 5%, the percent mortalities for all concentrations in the assay were transformed using abbot's formula (1). If control mortality exceeded 10%, mortality data were discarded and the assay was repeated. Mortality data were analyzed using the log-probit regression function in SPSS version 20. Treatments were considered significantly different from each other when the ratios of their $LD_{50/95}$ had confidence limits that excluded one.

RESULTS

Larval exposure to DTR had an effect on the susceptibility of adult mosquitoes to the insecticides tested for both the BZ and TH strains, though not always significant (Table 3). Both strains showed similar trends in susceptibility to malathion and propoxur but not permethrin. For permethrin increasing DTR resulted in a non-significant increase in adult susceptibility for the BZ strain and a significant decrease in adult susceptibility

for the TH strain (Figure 5). For the TH strain, the 15°C cohort exhibited a 4-fold increase, and the 20°C cohort 1-fold increase in $LD_{50/95}$ concentrations compared to both the 0°C and 10°C cohorts. As a result, dose-response curves for the TH strain generally shifted to the right with increasing DTR. For propoxur (Figure 6) and malathion (Figure 7) the general trend was for increased susceptibility with large DTR exposure, causing a general shift to the left in dose-response curves compared to 0°C cohort controls—though some variation was present. This was most evident in the BZ 15°C and 20°C cohorts exposed to malathion and the TH 15°C and 20°C cohorts exposed to propoxur, where both LD_{50} and LD_{95} concentrations were significantly lower than that of their respective 0°C cohorts. Interestingly, the 10°C cohort for both strains exhibited decreased susceptibility to both compounds but results were only significant in the BZ 10°C cohort exposed to propoxur and the TH 10°C cohort exposed to malathion.

DISCUSSION

In general, the TH strain appears more susceptible to the insecticides tested than the BZ strain. For permethrin, $LD_{50/95}$ values were two to 10-fold higher for the BZ strain across all four DTRs. The control groups showed the widest disparity between the two strains with a greater than10-fold difference. While this suggests possible resistance in the BZ strain, it is important to note that the 0°C cohort LD_{95} of 716.54 ng/cm^2 equates to a field dose of 7.2 mg/m^2, well below WHO recommended application rates of 20-30 mg/m^2 for pyrethroids in general. Increasing DTR reduced this disparity between the BZ and TH strains to a three-fold difference in LD_{95} values between the 15°C treatment groups. The BZ and TH stains also differed in their susceptibility to propoxur and malathion, but the magnitude of these differences were far less dramatic than for

permethrin, generally staying below a one-fold difference. For both strains, a small DTR of 10°C during larval development decreased susceptibility to both propoxur and malathion in the adult stage while DTRs larger than 10°C increased adult susceptibility. However, the increases observed in the 10°C cohorts were on the order of 0.15 mg/m^2 at the most, not near enough to push the 0.2-0.3 mg/m^2 0°C cohort rates above WHO recommended application rates of 2 g/m^2 for malathion or 1-2 g/m^2 for propoxur.

The fact that responses for both propoxur and malathion were congruent is not surprising given that they both target the same system. Interestingly, responses to permethrin were different than for propoxur and malathion for both strains but for different reasons. Belize mosquitoes showed a steady increase in susceptibility out to the largest DTR, 20°C, where a slight decrease from the previous DTR of 15°C was detected. For the TH strain, however, the 15°C and 20°C treatment groups exhibited a significant decrease in susceptibility. Interestingly, in Thailand, it has been shown that *Ae. aegypti* is a more effective dengue virus vector when exposed to smaller DTRs (38). These data suggest that the Thailand strain may be more susceptible to permethrin-based indoor residual spraying under the same environmental conditions that make them more competent as dengue vectors.

Given the different responses to permethrin, which binds to axonal sodium channels, compared to both propoxur and malathion, whose modes of action inhibits acetylcholinesterase function, it is apparent that DTR exposure acts in system specific ways. It is possible that DTR exposure during larval development can induce the overproduction, or inhibit production of, necessary detoxification enzymes that could lead to changes in adult susceptibility to selected pesticides. It has been demonstrated

that genes that code for detoxification enzymes are linked to pyrethroid resistance in *Ae. aegypti* (61). Also, Marcombe et al. (41) showed that the overproduction of some detoxification enzymes could play a role in deltamethrin resistance in *Ae. aegypti* from Martinique. Perhaps the larval thermal environment influences equilibrium set points for these enzyme systems in the adult stage.

Raghavendra et al. (55) found that mosquito larvae exposed to brief periods of thermal stress exhibited adult LT_{50} times to 5% malathion impregnated bed nets that were one-three fold longer than larvae reared under standard conditions. However, our data indicate that prolonged exposure to thermal stress may actually increase susceptibility to malathion, as evidenced by the reduction in $LD_{50/95}$ values at the more extreme DTRs of 15°C and 20°C. Despite this, under a 10°C regimen, susceptibility was reduced. However, it must be noted Raghavendra et al. (55) examined the effects of thermal stress on *An. gambiae* so the seemingly contrary results obtained here using *Ae. aegypti* may simply be due to differences between the species. Also, their work focused on lethal knockdown time instead on lethal dosage and we were not able to assess knockdown times due to the limitations of the current study. In order to have appropriate 24-hour mortality data for the construction of dose-response curves concentrations were selected that did not provide knockdown data appropriate for analyzing the effect of larval DTR exposure on knockdown times. Future work is needed to assess this effect using set diagnostic doses.

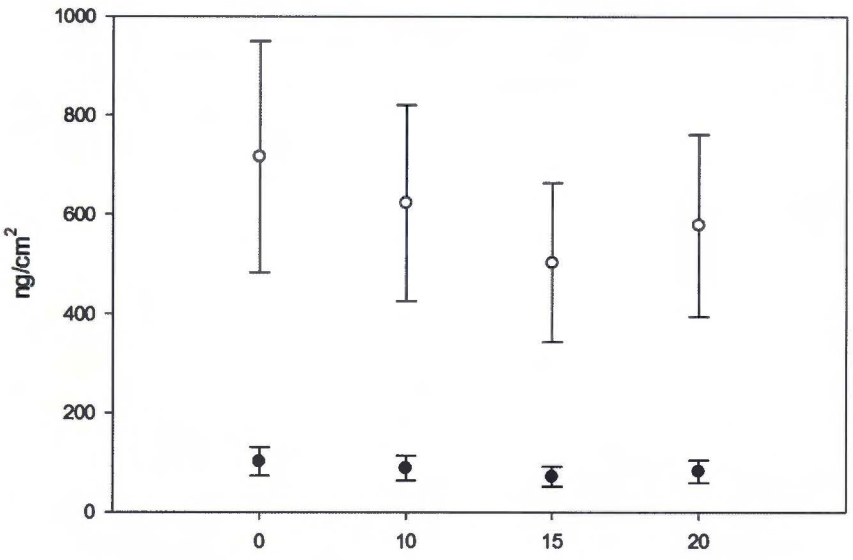
In this study, we showed that larval DTR exposure can influence the degree of adult susceptibility to insecticides in *Ae. aegypti*. However, adult susceptibility was not altered enough to impact WHO recommended field application rates for the

insecticides tested. Despite the affect larval DTR exposure had on adult susceptibility, all LD_{95} values were well below standard field application rates. Interestingly, the degree and direction of change in susceptibility appears to be target site specific with larval DTR exposure influencing adult susceptibility in different ways depending on the insecticide mode of action. For insecticides that target acetylcholinesterase, DTR exposure appears to decrease susceptibility under mild conditions (10°C) while more extreme exposure (15°C and 20°C) results in increased susceptibility. For permethrin DTR effects were strain specific. For the TH strain, the increase in DTR exposure resulted in significant decreases in susceptibility. In the BZ strain, however, susceptibility increased with increasing DTR exposure. While DTR exposure during larval development may not alter susceptibility enough to affect the efficacy of field application rates, these results are enough to suggest that resistance assays that utilize adult mosquitoes reared under standard laboratory conditions may be of little relevance in the field, where mosquito development occurs under natural conditions that include significant daily temperature fluctuations. Also, studies using field application of insecticides to non-sterile surfaces are needed to determine whether changes in susceptibility reported here could affect the efficacy of standard field application rates when these compounds are applied to representative field surfaces. In addition, further work is needed to determine what, if any, affect larval DTR exposure has on adult time to knockdown for commonly used insecticides. Furthermore, broad genetic/transcription analyses would help determine how thermal exposure in the larval stage influences expression of phenotypic traits in the adult.

Table 3. LD_{50} and LD_{95} values calculated from dose-response curves using log-probit regression methods for all chemicals tested across strains. Sample sizes for all curve estimations were 270-360 mosquitoes per cohort tested. Significance (*) was determined by comparing the ratios of LD_{50} and LD_{95} values between the 0°C control assays and each treatment. If the upper and lower 95% confidence intervals did not include one the difference between control and treatment was deemed significant. Dosage units for the $LD_{50/95}$ values are in ng/cm^2.

		Permethrin		**Propoxur**		**Malathion**	
Strain	**Treatment**	**LD_{50}**	**LD_{95}**	**LD_{50}**	**LD_{95}**	**LD_{50}**	**LD_{95}**
Belize	**0**	101.973	716.539	8.946	21.488	16.514	27.245
	10	88.639	622.845	15.105*	36.28*	18.941	31.248
	15	71.506	502.451	9.374	22.516	13.424*	22.146*
	20	82.279	578.151	7.724	18.552	13.175*	21.735*
Thailand	**0**	7.539	39.268	7.925	21.304	9.636	22.474
	10	8.581	44.696	8.82	23.709	13.892*	32.399*
	15	34.025*	177.222*	5.2*	13.978*	9.447	22.033
	20	15.73*	81.931*	5.017*	13.486*	10.705	24.966

a.



b.

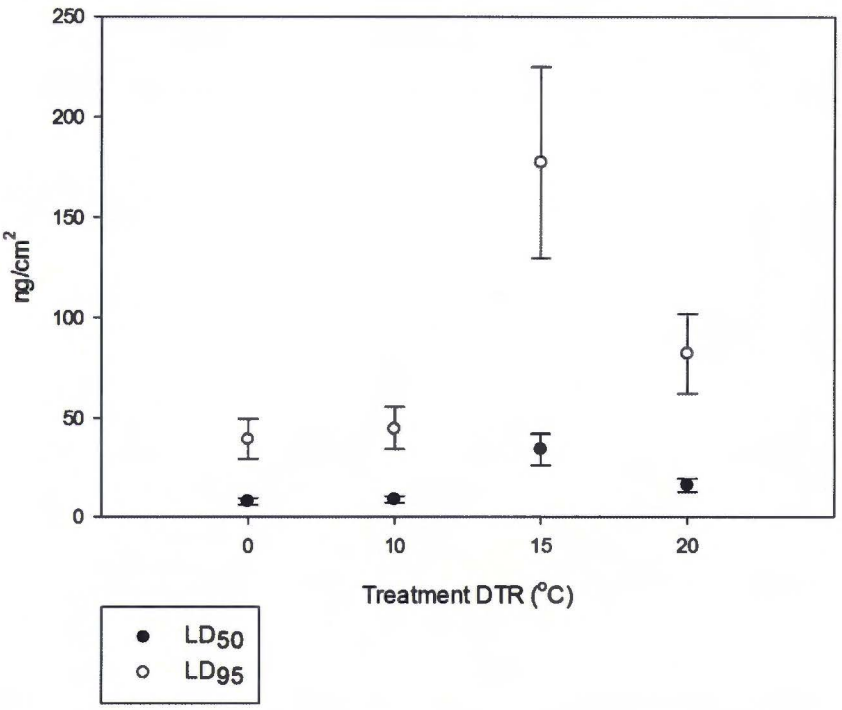
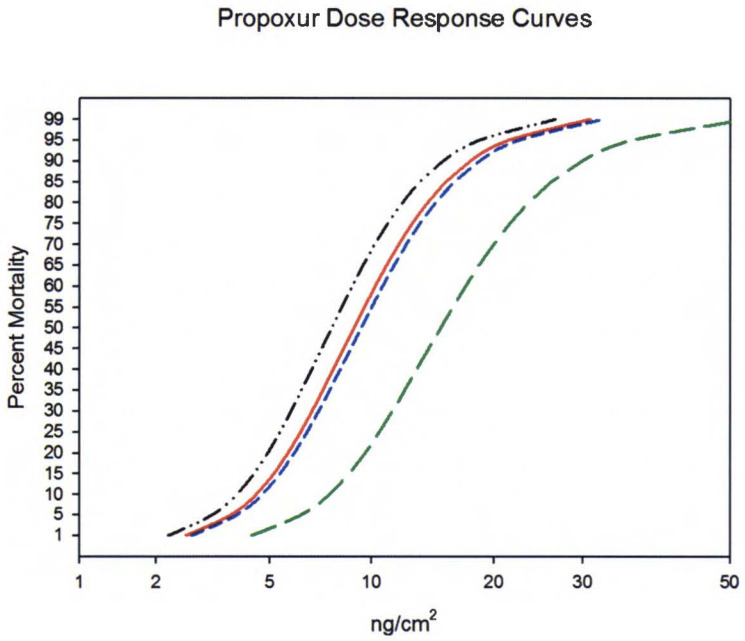


Figure 5. Probit estimated LD_{50} and LD_{95} concentrations for permethrin 24 hours post exposure for the Belize (a) and Thailand (b) strains of *Aedes aegypti*. Bars indicate 95% confidence intervals.

a.



b.

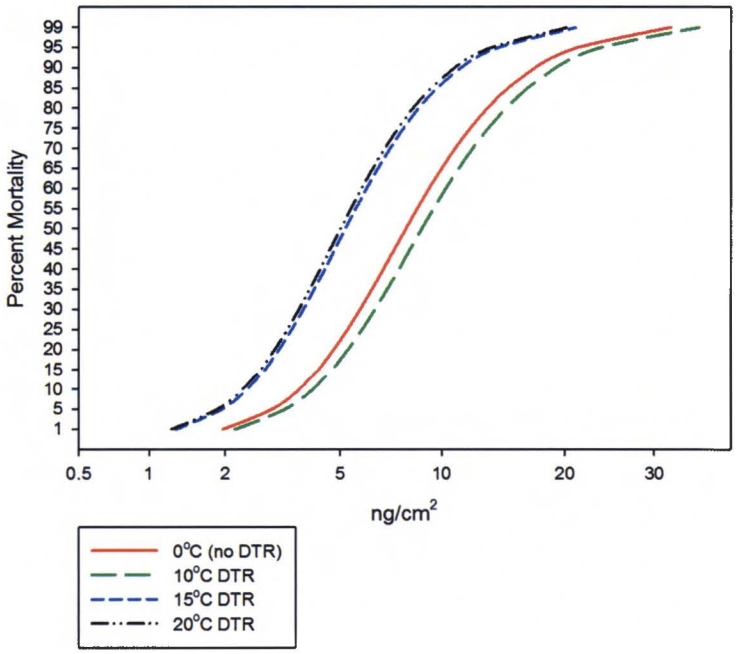
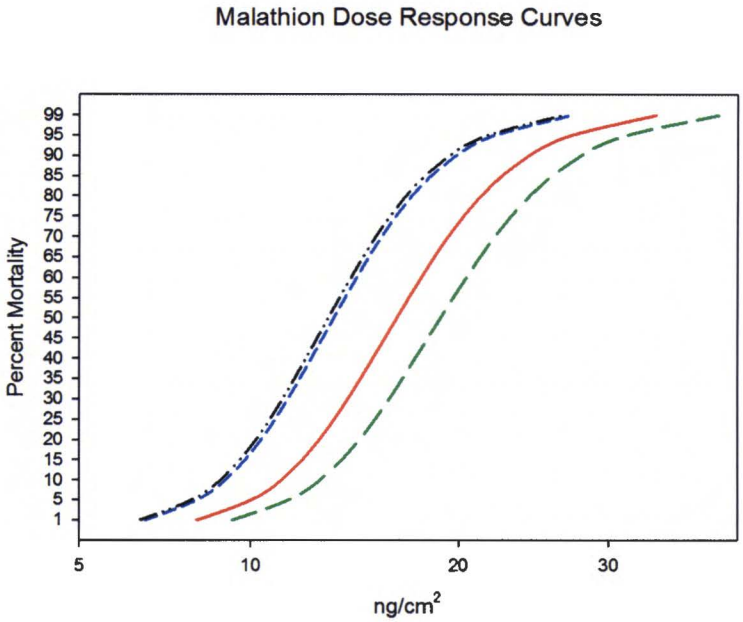


Figure 6. Twenty four hour post-exposure propoxur dose-response curves for Belize (a) and Thailand (b) *Aedes aegypti* cohorts. Values on the x-axis represent bottle propoxur AI residuals in ng/cm^2. The y-axis is the percent mortality predicted by the probit model.

a.



b.

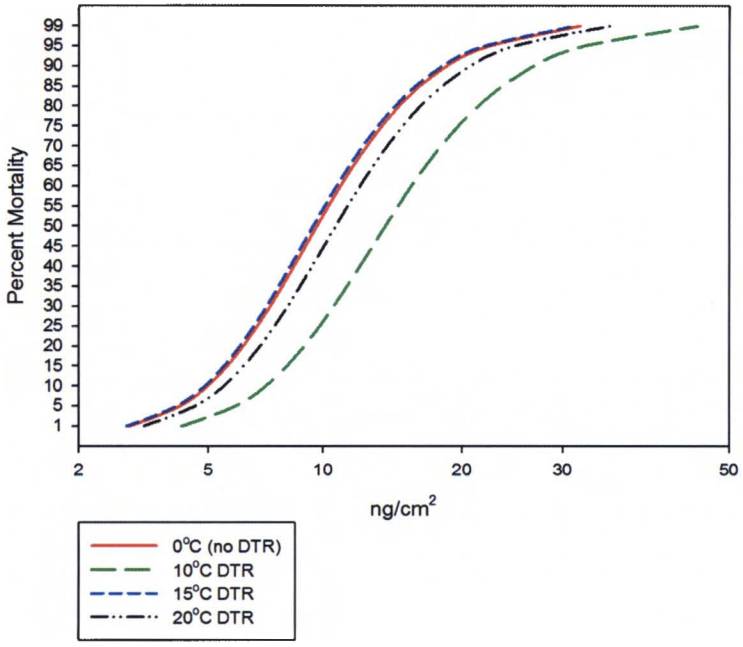


Figure 7. Twenty four hour post-exposure malathion dose-response curves for Belize (a) and Thailand (b) *Aedes aegypti* cohorts. Values on the x-axis represent bottle malathion AI residuals in ng/cm^2. The y-axis is the percent mortality predicted by the probit model.

CHAPTER 4: Influence of Larval DTR Exposure on Select Life History Traits in the Malaria Vector, *Anopheles gambiae* Patton.

AB STRACT

The general consensus is that the possession of an insecticide resistance mechanism has negative consequences in an insecticide-free environment. Also, several environmental variables are known to influence adult susceptibility to insecticides, including larval nutritional status and water quality. These variables have also been shown to affect adult life history traits such as survival and fecundity. If possession of a resistance phenotype is more costly in an insecticide free environment one would expect environmental stress to have a proportionately more negative impact on measured traits in a resistant strain, as compared to a wild type strain. The influence the larval thermal environment has on the expression of important life history traits between a susceptible and resistant insect population has never been considered. In this study we looked at how daily fluctuations around a standard mean temperature during larval development affected select life history traits in the malaria vector, *Anopheles gambiae*. To do this, we reared cohorts of the G3 type strain and the carbamate resistant AKRON strain under four daily treatment regimens ranging from 0°C to 20°C daily fluctuations around a standard mean rearing temperature of 28°C. Results suggest that larval exposure to diurnal temperature fluctuations in this species can severely limit reproductive output as measured by egg production. Increasing temperature fluctuations also significantly impacted pupation rates in the AKRON strain while only affecting the G3 strain at the extreme. These treatments had no influence on emergence rates for the AKRON strain while significant reductions in emergence were detected in the G3 strain. Sucrose

survival times increased significantly for the AKRON strain but decreased in the G3 strain with increasing diurnal temperature range exposure. Blood fed and starved survival rates, however, were unaffected. These results suggest that larval thermal exposure can have strain specific effects on adult characteristics, and that possession of a physiological resistance trait does not pre-dispose An. gambiae to reduced life history trait performance compared to wild type strains when exposed to sub-optimal environments.

INTRODUCTION

Insecticide resistance, or a decrease in the degree of susceptibility to a given concentration of an insecticide, is generally considered to be an evolutionarily expensive trait that imposes fitness costs in a pesticide-free environment. Most commonly, resistance is thought to be solely the result of physiological and/or behavioral modifications that lead to lack of target site binding, increased detoxification activities, increased sequestration, or avoidance of contact with the chemical (16, 30, 60). However, relatively little has been reported on the actual costs imposed on a resistant phenotype (3). Insecticide resistance costs reported to date include reductions in pre-imaginal survival (9, 22), decreased fecundity (25), reduced longevity (3, 13), mating competition costs (10), and increased predation costs (11). Additionally, Rivero et al. (58) showed that, on average, resistant *Cx. pipiens* emerged as adults with 30% less energetic reserves than susceptible mosquitoes. Interestingly, this cost was incurred during metamorphosis, as there was no difference in energetic reserves among resistant and susceptible fourth instar larvae. The quantity of energetic reserves accrued during larval

development has also been suggested to influence several factors that influence vector competence and vectorial capacity (59).

Coustau et al. (21) noted that, while some authors presented evidence of resistance associated fitness costs, others were unable to detect a cost. They suggested that resistance costs might only become apparent under a specific set of environmental conditions. Also, while the genetic determinants of insecticide resistance have been elucidated (14, 26, 69), the plasticity of phenotypic expression, to my knowledge, has never been examined. Bourguet et al. (14) did examine the plasticity of resistance allele dominance and found that the dominance of resistance expression (due to an insensitive acetylcholinesterase allele in *Cx. pipiens*) was dependent on the environmental variables examined: larval density, container depth, and daylight length. They showed that the expression of resistance in a heterozygous population was associated with optimal environments. In other words, resistance heterozygotes expressed a resistant phenotype only under optimal rearing conditions. Given this, it is reasonable to assume that abiotic environmental variables could influence the cost and expression of insecticide resistance in a given population.

Among these variables is the thermal environment in which an insect must function. Ambient temperature can have a huge influence on physiological processes in cold blooded animals. For instance, warmer temperatures have been shown to increase hemolymph pH in the locust *Schistocerca nitens* (29) and speed hemolymph acidosis recovery rates in the grasshopper *Melanoplus bivittatus* (28). Also, ambient temperature is known to be an important constraint on the immune response (12) while small changes in ambient temperature have the ability to influence the outcome of insect-parasite

interactions (65). Indeed, Benelli (8) showed that this influence can be carried over to the adult stage as larval exposure to low temperatures reduced immune encapsulation responses in adults.

In addition, ambient temperature has been shown to influence measured life-history traits in vector populations (4, 39, 57) as well as alter disease transmission dynamics. For example, Westbrook et al. (70) showed that rearing *Ae. albopictus* at 18°C produced larger females that were six times more likely to be infected by Chikungunya virus (CHIKV) than when reared at 32°C. Additionally, Watts et al. (68) showed that, while *Ae. aegypti* could become infected with dengue virus (DEN-2) at any temperature from 20°C to 35°C, only those mosquitoes held at 30°C or higher were able to subsequently transmit the virus to monkeys, regardless of infectious dose. However, the influence ambient temperature has on vector/pathogen interactions is not consistent across systems. For instance, higher temperatures increase infection rates of Rift Valley fever virus in *Cx. pipiens* (67) while decreasing infection rates of Eastern Equine Encephalitis virus in *Cx. tarsalis* (36).

In holometabolous disease vectors it is not enough to consider ambient temperature effects on the adult population alone, but consideration should also be given to influences on immature development and how these influences affect adult characteristics. Mosquito breeding sites are continually exposed to the vagaries of changing climactic conditions. This includes exposure to changing mean temperatures caused by cyclical climactic patterns, such as the el-nino southern oscillation, as well as short-term cyclical changes in ambient temperature caused by solar warming during the day and cooling at night. In terms of mosquito biology/physiology it can be argued that

short-term daily fluctuations in temperature can influence mosquito life history traits much more drastically than shifts in long-term mean ambient temperature, whether over the course of weeks, months, or years. These long-term shifts provide evolutionary forces a chance to shape the phenotype through continued fine-tuning of optimal life history traits through successive generations. However, exposure to daily cyclical changes has the potential to influence the outcome of life history traits of individuals within a single generation. This could lead to differing outcomes for the same measured traits from intra-generational cohorts exposed to differing conditions (phenotypic plasticity) leading to greater observed variability of these traits in a natural population. As a result, natural populations may be better able to cope with long term shifts in mean temperature than may be presumed based on fitness estimates from lab reared cohorts. Indeed, Long (40) suggested that overall population fitness tends to be greater in environments with more frequent fluctuations in temperature while Beardmore and Levine (5) showed that diurnal temperature fluctuations produce *Drosophila psuedoobscura* (Frolova) larvae with higher viability.

Despite this, the majority of disease vector work to date has focused on the role of static mean temperatures on pathogen-vector systems. However, the influence daily temperature fluctuations, or the diurnal temperature range (DTR), has on these systems have only recently been the subject of some inquiry. Lambrechts et al. (38) showed that *Ae. aegypti* from Thailand were less susceptible to dengue virus infection and exhibited faster mortality at larger DTRs around the same mean ambient temperature. In doing so, they showed that the defining predictive factor for dengue virus transmission in Thailand might not be mean seasonal temperature or rainfall but the size of the DTR around the

mean daily temperature. Additionally, Paaijmans et al. (53) showed that fluctuation in diurnal temperatures reduces the impact of increasing mean temperatures when using a thermodynamic model for malaria development (model parameters 18-28°C). Specifically, they showed that diurnal temperature fluctuations around means >21°C slow parasite development while fluctuations around means <21°C increases parasite development. Expanding on this model, Paaijmans et al. (50) showed that, at the model extremes, diurnal temperature fluctuations makes transmission possible at lower mean temperatures and reduces transmission at higher mean temperatures than that predicted by the standard static mean temperature models. As a result, current models for predicting malaria transmission risk may underestimate transmission at the fringes of endemic zones, such as in the Highlands of East Africa, while, at the same time, overestimating the risk in warmer portions of endemic zones (50).

While these investigations focused on the effect of DTR on adult mosquito parameters, field mosquito development sites are exposed to the same daily warming and cooling cycles that adult mosquitoes are exposed to. Adult mosquitoes, however, have the ability to moderate their exposure to daily temperature fluctuations through behavioral avoidance, e.g. responding to changes in microclimactic cues via the search and selection of more suitable resting locations. In contrast, exposure of mosquito larvae to diurnal temperature fluctuations is determined by the temporal characteristics of the development site. While these fluctuations may be minimal for species that breed in large bodies of water, species that utilize small ephemeral sites such as small puddles, tire ruts, hoof prints, discarded tires and water holding tanks are potentially much more exposed—especially if these locations receive direct sunlight for any portion of the day.

Several prominent disease vectors routinely utilize such habitats as breeding sites. *Anopheles gambiae* s.l. Patton, the principal vector of malaria in much of Africa, is known to utilize anthropogenic breeding sources such as tire ruts, hoof prints, and drainage channels (37, 45) as well as natural sources such as rain pools and burrow pits (45). In deforested areas these sites have been shown to be more productive in terms of adult mosquitoes produced (44) than those in forested areas, presumably due to the fact that direct exposure to sunlight increases mean water temperatures.

Given the exposure of some larval mosquito populations to diurnal changes in temperature, it is surprising that the effect of this exposure on mosquito development has scarcely been addressed. There is dearth of information on the temporal temperature profiles of field breeding and development sites in general. Standard practice for all research that involves the use of laboratory-reared mosquitoes utilizes mosquitoes that are reared at a constant temperature. The effect of exposure to diurnal temperature fluctuations during larval development has all but been ignored. In the prior experiments looking at the role of DTR on disease transmission, mosquito vectors were reared under standard rearing conditions with no fluctuations in temperature. In fact, Paaijmans et al. (52) showed that standard anopheline development sites in the field exhibited diurnal temperature fluctuations with a net effect of maintaining mean habitat water temperatures 4-6^{o}C higher than mean ambient temperatures. This difference resulted in faster development times than predicted based on mean ambient temperature alone. One of the few studies to date examining the effect of diurnal temperature fluctuations on the development of mosquito larvae showed a highly female skewed sex ratio at higher temperatures and a lack of correlation between adult body size and rearing temperature

(42). Indeed, larval exposure to diurnal temperature fluctuations in the field could well explain the results of Tun-Lin et al. (66) who showed that correlations between wing length and temperature in field populations of *Ae. aegypti* were much lower than that for laboratory-reared mosquitoes.

Carrington et al. (18) incriminated diurnal temperature fluctuations for this inconsistency when they examined the effect small (8°C) and large (18°C) diurnal temperature fluctuations have on select life-history traits in a strain of *Ae. aegypti* from Thailand. To date, this is the only work that explores the influence of larval exposure to diurnal temperature fluctuations on important adult life-history traits in a vector population. Through their work they showed that differing DTRs have an effect on immature development time, survival to adulthood and female reproductive output. In addition to the parameters evaluated by Carrington et al. (18) we also examined the influence of DTR on other parameters of epidemiological importance, such as blood-feeding frequency, fertility (estimated through egg viability rates), and survival rates for starved, glucose-fed and blood-fed cohorts in the malaria vector, *An. gambiae* s.s. In order to determine whether larval exposure to diurnal temperature fluctuations have differential effects on knockdown resistant mosquitoes we used two colonized strains of *An. gambiae* s.s., the wild type G3 strain originally from The Gambia and the AKRON strain with carbamate resistance originally from Benin.

METHODS

Mosquito Strains

Wild type G3 strain susceptible (F240) and carbamate resistant AKRON strain (F5) *An.* gambiae eggs were obtained from the Centers for Disease Control's Malaria

Research and Reference Reagent Resource Center (MR4) in Atlanta, GA. Eggs were used to establish laboratory colonies at the USUHS insectaries to serve as source stock for multiple experiments.

Rearing Conditions

All mosquitoes were reared using Percival I-36VL incubators (Percival Scientific, Perry IA). Briefly, eggs were hatched under standard rearing conditions (28°C, LD 12:12, RH 80%) and hatched larvae allowed to develop for 24 hrs. Larvae were then separated at the rate of 200 larvae per 18 x 25 cm collection tray containing 900ml dH_2O. Each larval tray was fed a total of 0.42g ground TetraFin® goldfish flakes (Tetra Holding, inc, Blacksburg, VA) over the course of larval development (0.045g on days 1, 3, 4, and 7; 0.12g on days 5 and 6). Trays were skimmed by lightly dragging a kim-wipe across the surface of the water on day 3 and on day 4 an additional 450 ml of dH_2O, warmed to incubator temperature, was added to each tray. Pupae were collected with disposable pipettes and placed in 500 ml emergence cups inside one gallon plastic buckets. Pupae and adults were maintained under standard rearing conditions (28°C, LD 12:12, RH 80%) and provided 10% sucrose cotton pledglets ad libitum. Larval cohorts from each strain were reared under one of four daily temperature treatments (LD 12:12, RH 80%) from 24hrs post hatch to pupation. Temperature treatments consisted of a DTR of 0°C, 10°C, 15°C and 20°C around a mean 28°C. Daily temperature curves (except for the 0°C range) followed a truncated sine wave progression during the light phase (day) and a decaying exponential progression during the dark phase (night). These curves have been found to accurately mimic diurnal variation in soil and air temperature during the course of a solar day (54). One cohort from each strain was reared under each DTR

treatment for a total of 8 cohorts for use in the following experiments. Both air and water temperature profiles were monitored with HOBO data loggers (Onset, Cape Cod, MA). Mean daily air temperature ranged from 27.4°C to 28.1°C while mean daily water temperature ranged from 26.7°C to 27.4°C across all treatments. All treatment daily mean temperatures were within 0.2°C of each other except for those cohorts reared under a 20°C DTR, which were, on average, 0.5°C cooler representing the lower end of the range.

Developmental Success and Survival Evaluations

To estimate the effect the four DTRs had on immature development and adult longevity the following experiments were conducted. For immature developmental success (percent pupation and percent emergence) and sex ratio, 200 one-day old mosquito larvae were separated and reared according to the above treatment protocols. Pupae were counted and collected as above, date of pupation recorded, and allowed 5 days to emerge. At the end of the 5 days adult containers were placed in a -20°C freezer and left overnight. The following day the dead mosquitoes were sorted by sex and counted. For the creation of survival curves, groups of 30 male and 30 female mosquitoes were separated for each cohort. These sub-cohorts were set up 24hrs post-emergence in one gallon plastic buckets with netted lids and held under standard rearing conditions (28°C, LD 12:12, RH 80%). Mosquitoes were given access to either 10% sucrose solution or water via soaked cotton pledglets, ad libitum, depending on the treatment (sugar-fed vs. starved). Pledglets were placed on the netted lids and refreshed daily at the same time mortality was recorded. Blood fed survival analyses were based on daily mortality data collected from cohort isolines described below.

Female Isolines

To estimate the influence DTR has on mosquito fecundity, gonotrophic cycle, blood feeding frequency, and percent egg hatch 30 female isolines were set up for each mosquito cohort. Three-day old adult buckets of 250 mixed sex adults were provided a bloodmeal and allowed to rest/mate for 24 hours. Individual engorged females were then placed in 40-dram plastic collection vials (Bioquip inc., Ranch Dominguez, CA) via gentle aspiration. Vial lids were modified by cutting out a center circle 3cm in diameter and gluing untreated mosquito netting in place with a hot glue gun. Before placing a mosquito in each vial an oviposition funnel was made from 9 cm round filter paper (Fisher Scientific, Pittsburgh, PA) and placed at the bottom of the vial with a small amount of dH_2O. Isolines were maintained under standard rearing conditions (28°C, LD 12:12, RH 80%) and each female given the opportunity to blood feed for 10 minutes every Monday, Wednesday, Friday, and Saturday. Mosquitoes still feeding at the end of the 10 minute interval were allowed to feed to repletion. Isolines were provided 10% sucrose pledgelets when not being offered a blood meal. Mosquitoes that laid eggs on non-blood feeding days (Tuesday, Thursday, and Sunday) were given a 10-minute opportunity to take a bloodmeal. After blood feeding, mosquitoes that oviposited eggs were transferred to a new 40-dram vial with a fresh oviposition funnel. Eggs were counted and prepared for hatch. After counting, dH_2O was added to cover the filter paper containing eggs and a small amount of food added. Eggs were then left to hatch in an incubator (28°C, LD 12:12, RH 80%) for 48hrs before the number of hatched larvae were counted. The length of the gonotrophic cycle was measured as the number of days between the first blood meal and the first eggs laid. This first blood meal after the eggs in a cycle were deposited was considered the start of a new gonotrophic cycle.

Interestingly, mosquitoes that had started, but not finished, laying a clutch of eggs when the next blood meal was offered refrained from feeding.

Statistics

All comparisons focused on analyzing differences between the 0°C cohort and all other cohorts for each strain. Developmental success rates were compared using binary logistic regression to obtain chi square scores for each comparison. Number of larvae that failed to pupate was found by subtracting the total number of pupae from the starting cohort number. The number of pupae that failed to emerge was found by subtracting the total number of adults that emerged from the total number of pupae for their respective cohorts. Survival analyses were compared using Kaplan-Meier methods with one time unit equal to one day and case data weighted by the number of mosquitoes that died on each day. Female isoline data were compared using one-way ANOVA analyses for each strain.

RESULTS

Developmental Success

Diurnal temperature fluctuations significantly affected percent pupation rates in the G3 strain (Wald χ^2= 22.487, DF= 3, p<0.001) but not the AKRON strain (Wald χ^2 = 6.778, DF= 3, p=0.079). For G3 cohorts, increases in DTR resulted in small decreases in pupation rates with significant differences between the 20°C cohort compared to the 0°C control (0°C= 98%; 10°C DTR= 97%, Wald χ^2 = 0.093, DF= 1, p= 0.76; 15°C= 94%, Wald χ^2 = 2.831, DF= 1, p= 0.092; 20°C= 87%, Wald χ^2 = 13.156, DF= 1, p< 0.001). While the overall model for the AKRON strain was not significant when comparing all treatments to the 0°C control, as with the G3 strain, pupation rates tended to decrease

with increasing DTR (Figure 8), with the 15°C cohort pupation rate significantly lower than that of the 0°C control cohort (0°C= 86%; 10°C DTR= 82%, Wald χ^2 = 1.185, DF= 1, p= 0.276; 15°C= 76%, Wald χ^2 = 6.369, DF= 1, p= 0.012; 20°C= 80% Wald χ^2 = 2.93, DF= 1, p= 0.087). This trend was in the same direction for both strains but AKRON pupation rates were significantly lower than those of the G3 strain (Wald χ^2 = 55.119, DF= 1, p< 0.001). As with pupation rates, adult emergence from collected pupae was significantly reduced by DTR treatment in the G3 strain (Wald χ^2= 20.169, DF= 3, p<0.001) but not the AKRON strain (Wald χ^2 = 4.363, DF= 3, p=0.225). Percent emergence among G3 cohorts declined with increasing DTR, becoming significant at 15°C and 20°C (0°C= 98%; 10°C DTR= 97%, Wald χ^2 = 0.119, DF= 1, p= 0.731; 15°C= 94%, Wald χ^2 = 4.047, DF= 1, p= 0.044; 20°C= 87% Wald χ^2 = 12.244, DF= 1, p< 0.001) with a similar pattern seen for the AKRON strain. While overall emergence rates were not significantly affected by DTR in the AKRON strain there was a slightly significant reduction in emergence in the 10°C cohort (0°C= 94%; 10°C DTR= 88%, Wald χ^2 = 4.032, DF= 1, p= 0.045; 15°C= 89%, Wald χ^2 = 2.948, DF= 1, p= 0.086; 20°C= 90% Wald χ^2 = 2.014, DF= 1, p= 0.156). As with pupation rates, AKRON emergence rates were significantly lower than that in the G3 strain (Wald χ^2 = 7.768, DF= 1, p= 0.005).

Diurnal temperature fluctuations did not appear to have an effect adult sex ratios in the G3 strain with ratios ranging from 0.9:1(male:female) in the 10°C cohort to 1.2:1 in the 15°C cohort. Both 0°C and 20°C cohorts in the G3 strain had ratios of 1:1. The AKRON strain saw a shift from a male skewed sex ratio of 1.2:1 in the 0°C cohort to female skewed ratios of 0.7:1, 0.9:1 and 0.7:1 in the 10°C, 15°C, and 20°C treatment cohorts, respectively.

Survival Analyses

Mean sucrose-fed adult female survival times ranged from 13.9 to 24.4 days across treatments for the G3 strain (pooled cohorts mean ± SE = 19.7 ± 0.525), whereas that for the AKRON strain ranged from 17.8 to 29.4 days (pooled cohorts mean ± SE = 21.9 ± 0.542). Male mean survival time ranged from 17.1 to 23.9 days for G3 cohorts (pooled cohorts mean ± SE = 20.9 ± 0.468) and 23.9 to 28.5 days for AKRON cohorts (pooled cohorts mean ± SE = 26.0 ± 0.536). The comparison of survival curves across treatments within each strain using log-rank tests showed that increasing DTR led to a significant reduction in female survival times for the G3 strain while leading to a significant increase in the AKRON strain (Table 4).

Increasing DTR caused significant decreases in G3 strain longevity for the 15°C and 20°C cohorts (χ^2= 45.403, DF= 3, p< 0.001 and χ^2= 49.906, DF= 3, p< 0.001, respectively) while causing significant increases in AKRON strain longevity for the 10°C, 15°C and 20°C cohorts (χ^2= 7.924, DF= 3, p= 0.005; χ^2= 51.902, DF=3, p< 0.001 and χ^2= 8.944, DF= 3, p= 0.003, respectively). Larval DTR exposure similarly affected male cohorts in the same strain-specific manner (Table 4) with a significant decrease in longevity detected in the G3 15°C and 20°C cohorts (χ^2= 7.27, DF= 3, p= 0.007 and χ^2= 48.605, DF= 3, p< 0.001, respectively), and a significant increase detected in the AKRON 10°C and 15°C cohorts (χ^2= 3.911, DF= 3, p= 0.048 and χ^2= 10.118, DF= 3, p= 0.001, respectively).

Blood-fed survival in both the G3 and AKRON cohorts was generally not affected by increasing DTR (Table 4). Means for these cohorts ranged from 28.7 to 31.9 days (pooled cohorts mean ± SE = 30.5 ± 0.814) for the G3 strain and 31.0 to 34.9 days (pooled cohorts mean ± SE = 32.8 ± 0.035) for the AKRON strain. Mean starved

longevity ranged from 4.1 to 4.5 days for females (pooled cohorts mean ± SE = 4.3 ± 0.048) and 3.7 to 4.6 days for males (pooled cohorts mean ± SE = 4.2 ± 0.069) in the G3 strain while in the AKRON strain starved longevity ranged from 3.9 to 5.4 days for females (pooled cohorts mean ± SE = 4.9 ± 0.081) and 4.0 to 5.4 days for males (pooled cohorts mean ± SE = 4.9 ± 0.075). When significant differences in starved longevity were detected between the DTR cohorts and the 0°C controls in each strain, it was always in the negative direction (Table 4).

Female Isolines

One-way ANOVA analysis revealed that larval DTR exposure had a significant impact on all life history traits measured in the G3 strain except for the number of blood meals taken per gonotrophic cycle and longevity (Table 5). Egg production and gonotrophic cycle traits could not be analyzed for the AKRON strain due to a general lack of egg production across all four DTR cohorts. For the remaining life history traits a significant difference among AKRON DTR cohorts was detected, except in the case of longevity (Table 5).

Increasing DTR caused significant reductions in the lifetime number of blood meals taken by females in both the G3 and AKRON strains. Total number of blood meals taken by G3 strain females decreased from 4.5± 0.577 (±SE) in the 0°C cohort to 2.4± 0.141 (±SE) in the 20°C cohort. In the AKRON strain, mean blood meals taken decreased from 3.8± 0.307 (±SE) in the 0°C cohort to 1.7± 0.175 (±SE) in the 20°C cohort. Time between blood meals in the G3 strain significantly increased from 7.5± 0.718 days in the 0°C cohort to a peak of 12.8± 1.129 (±SE) days in the 15°C cohort before coming back down slightly to 10.6± 0.956 (±SE) in the 20°C cohort . In the

AKRON strain the time between blood meals increased significantly from 9.1± 1.337 (±SE) days in the 0°C cohort to 18.1± 2.652 (±SE) days in the 20°C cohort.

For the G3 strain the total number of gonotrophic cycles decreased significantly with increasing DTR from 3.0± 0.694 (±SE) in the 0°C cohort to 0 in the 20°C cohort. However, while not significant, the number of blood meals taken per gonotrophic cycle increased from 1.3± 0.133 (±SE) in the 0°C cohort to 1.7± 0.145 (±SE) in the 15°C cohort. It appears that this increase may be attributed to the significantly increased length of the gonotrophic cycle detected with increasing DTR treatments. Gonotrophic cycle length increased from 5.8± 0.839 (±SE) days in the 0°C cohort to 22.6± 8.743 (±SE) days in the 15°C cohort. Increasing DTR also caused significant reductions in egg production in the G3 strain to the point where no eggs were produced by any G3 females in the 20°C cohort (Figure 9). Significant reductions were also seen in the percent of eggs hatching for each cohort with 62% hatching in the 0°C control, 36% in the 10°C cohort, and 8% in the 15°C cohort.

DISCUSSION

Based on these results it is evident that the magnitude of diurnal temperature fluctuations in *An. gambiae* larval development sites influences multiple characteristics in post-larval life stages in this mosquito. Pupation and emergence rates decreased with increasing DTR with the carbamate resistant AKRON strain exhibiting pupation and emergence rates that were consistently lower than those of the susceptible G3 strain regardless of DTR. It is possible that the metabolic cost of carbamate resistance is such that, under identical circumstances, meeting the metabolic reserve requirements for

successful pupation and emergence is more difficult, thus leading to decreased rates compared to the susceptible G3 strain.

Interestingly, mean survival times in the AKRON strain were longer than the G3 strain for the majority of DTR cohorts. Larval DTR exposure led to increased sucrose survival times for AKRON male and female cohorts while decreasing survival rates in G3 cohorts of both sexes. All male and female AKRON cohorts had longer survival times than corresponding G3 cohorts except for 0°C and 10°C females. No real trend was detected among male and female starved cohorts and all AKRON cohorts exhibited higher survival rates than susceptible G3 cohorts, except at 20°C for both males and females. Survival rates among blood fed AKRON females in the 0°C and 10°C cohorts were, on average, three days longer than G3 females in these cohorts. Survival times in the 15°C and 20°C G3 cohorts increased to match (15°C cohort) or exceed (20°C cohort) survival times in the AKRON strain. This increase in survival time among blood fed G3 females corresponds to the decrease in egg production that was observed with increasing DTR in the G3 strain.

Female AKRON strain isolines exhibited severely reduced egg production as adults (total of 166 eggs produced in three clutches by 2 females out of 120 female isolines). As a result, these isolines were not subjected to the repeated metabolic stresses encountered during repeated egg production cycles. Given this, it is possible that the differential in blood fed survival rates with 0°C and 10°C G3 cohorts exhibiting lower survival rates, and the 15°C and 20°C G3 cohorts exhibiting similar or slightly increased survival rates, compared to the corresponding AKRON cohorts, could simply be a

function of cohort egg production rates and thus not directly attributable to the larval DTR treatment regime alone.

The lack of egg production by the AKRON cohorts may have influenced other variables as well. For instance, the G3 cohorts took more blood meals than the AKRON cohorts despite having decreased survival in the 0°C and 10°C cohorts. Indeed, the total number of blood meals taken by each G3 cohort decreased with increasing DTR treatment despite increased survival in the 15°C and 20°C cohorts. While DTR appears to influence these traits, based on our results we cannot rule out that the observed shifts in blood feeding behavior were simply due to the decrease or lack of egg production in these strains. Since the decrease in blood feeding behavior in the G3 strain corresponded with the gradual shut down of egg production with increasing DTR it is possible that DTR has an indirect effect on *An. gambiae* blood feeding behavior through the inhibition of egg production with larger DTRs. Egg production steadily decreased from a mean lifetime production of 263eggs per female (average clutch size of 87 eggs per cycle) in the 0°C cohort to 0 in the 20°C cohort. Furthermore, the proportion of females actively producing eggs steadily decreased with increasing DTR from 60% in the 0°C cohort to 0% in the 20°C cohort (Figure 3). In addition, the length of time (in days) until the completion of the first oviposition cycle increased with increasing DTR (Figure 2). This shows that exposure to large DTRs during larval development in this species can severely restrict egg production in the adult stage. While we could not analyze the effect larval DTR exposure had on egg production in the AKRON strain, the three AKRON females that did produce eggs were limited to the 0°C and 10°C cohorts.

These results demonstrate that larval DTR exposure can have a differential impact on pupation and emergence rates between susceptible (G3) and resistant (AKRON) mosquitoes in *An. gambiae*. Larval DTR exposure led to steadily declining pupation and emergence rates for each consecutive increase in DTR for the G3 strain. However, for the AKRON strain, emergence rates dropped when DTR exposure was present but were relatively unchanged between the three non-control DTR cohorts. It appears that resistant AKRON mosquitoes that make it to adulthood may experience higher longevity rates than susceptible G3 mosquitoes when resources are low as evidenced by the consistently higher survival rates in starved AKRON cohorts. When sugar meals are present, G3 mosquitoes exhibited better survival rates than AKRON mosquitoes at low DTR. However, AKRON mosquitoes were better able to cope with increasing DTR and exhibited higher survival rates at the more extreme DTR treatments.

Despite detecting some differential DTR trends between G3 and AKRON cohorts, it is difficult to say whether these differences can be attributed to the presence of the ACE-1 mutation conferring carbamate resistance. Separate work on two geographically isolated populations of *Ae. aegypti* suggests that the direction of trend with increasing larval DTR exposure may be population dependent and not static across a species (Chapter 2). Since the source populations for the G3 and AKRON strains are from different geographic locations source population genetic effects on the observed changes in these traits cannot be ruled out. The fact that the G3 strain is a long-established colony while the AKRON strain was only recently established further lends to confounding the interpretation of these results.

In this study we showed that the extent of larval exposure to diurnal temperature fluctuations can influence the expression of life history traits in adult *An. gambiae*. Most surprising was the shutdown of egg production by adult female mosquitoes exposed to large DTRs as larvae. What was striking about this was that it appears to be a graded response dependent on the degree of larval DTR exposure. This response also appeared to be the result of three interacting variables that coalesced to produce the observed result. Increases in larval DTR exposure caused not only a steady decrease in the number of eggs produced, but the number of females who deposited eggs steadily decreased and the length of time before their first oviposition episode steadily increased until all values were 0 at a DTR of 20°C. This suggests that, while *An. gambiae* can survive large diurnal fluctuations in the larval environment their ability to reproduce becomes severely restricted. It is possible that this inability to produce eggs after exposure to larger DTRs serves as a barrier preventing *An. gambiae* from effectively utilizing container breeding sites. Future work should focus on determining the physiological mechanisms causing this loss of egg production at higher DTRs and whether this is a common outcome in other *Anopheles* spp. as well. While we were unable to say for certain that larval DTR exposure differentially affects a kdr *An. gambiae* strain compared to a wild type strain, we were able to show that the AKRON strain was sometimes more severely affected and sometimes less severely affected than the G3 wild type strain depending on the trait being examined. However, more detailed studies are needed to tease out the effects larval exposure to diurnal temperature fluctuations have on kdr vs. non-kdr populations (as well as populations with other types of resistance) apart from contributing factors such as differences in source population composition and variability.

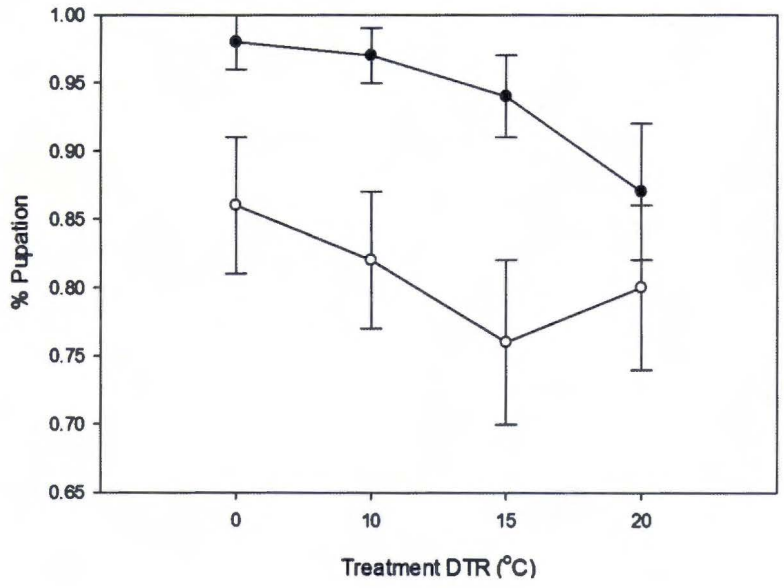
Table 4. Mean survival times ± standard errors (in days) with log-rank test results for pairwise comparisons of sucrose, blood-fed, and starved survival curves constructed using Kaplan-Meier methods. Tested DTRs were compared against the control 0°C DTR cohort for each strain. Sample size for each DTR cohort ranged from 30 to 35 mosquitoes each. For all pairwise comparisons DF= 3. * = significant at the p= 0.05 level.

		G3	AKRON
Sucrose-fed			
Female	**0°C**	23.79±1.19	17.76±0.73
	10°C	24.38±0.54, χ^2= 0.135, p= 0.714	21.29±0.69, χ^2= 7.924, p= 0.005*
	15°C	17.26±0.4, χ^2= 45.403, p < 0.001*	29.41±1.13, χ^2= 51.902, p< 0.001*
	20°C	13.89±0.52, χ^2= 49.906, p< 0.001*	21.17±0.67, χ^2= 8.944, p= 0.003*
Male	**0°C**	23.97±0.59	25.58±0.95
	10°C	23.73±0.92, χ^2= 0.172, p= 0.678	23.97±0.9, χ^2= 3.911, p= 0.048*
	15°C	19.41±0.91, χ^2= 7.27, p= 0.007*	28.57±1.28, χ^2= 10.118, p= 0.001*
	20°C	17.1±0.6, χ^2= 48.605, p< 0.001*	26.13±0.54, χ^2= 0.31, p= 0.578
Blood-fed			
Female	**0°C**	28.72±1.7	34.9±2.18
	10°C	29.32±1.61, χ^2= 0.063, p= 0.801	32.63±1.75, χ^2= 0.784, p= 0.376
	15°C	31.97±1.81, χ^2= 0.874, p= 0.35	32.57±1.23, χ^2= 4.269, p= 0.039*
	20°C	31.9±1.36, χ^2= 0.198, p= 0.656	31.0±2.2, χ^2= 2.448, p= 0.118
Starved			
Female	**0°C**	4.52±0.09	5.44±0.16
	10°C	4.4±0.09, χ^2= 0.803, p= 0.37	5.0±0.08, χ^2= 6.682, p= 0.01*
	15°C	4.14±0.1, χ^2= 6.862, p= 0.009*	5.47±0.09, χ^2= 0.048, p= 0.826
	20°C	4.26±0.09, χ^2= 3.513, p= 0.061	3.86±0.09, χ^2= 40.013, p< 0.001*
Male	**0°C**	4.57±0.09	5.38±0.14
	10°C	4.08±0.14, χ^2= 7.541, p= 0.006*	5.13±0.09, χ^2= 2.1, p= 0.147
	15°C	3.73±0.14, χ^2= 15.726, p< 0.001*	5.28±0.12, χ^2= 0.352, p= 0.553
	20°C	4.32±0.13, χ^2= 1.575, p= 0.209	4.0±0.1, χ^2= 40.013, p< 0.001*

Table 5. Results of one way ANOVA analyses comparing selected life-history traits between female isolines from mosquito cohorts reared at a constant 28°C to those reared under diurnal temperature fluctuations of 10, 15, and 20°C. Sample size for each cohort consisted of 30 female mosquitoes each. * = significant at the p= 0.05 level.

Strain	Trait	F Statistic	P Value
G3	Total # of blood meals	5.947, DF=3	P= 0.001*
	Blood feeding frequency	5.867, DF=3	P= 0.001*
	Total # of gonotrophic cycles	11.785, DF=3	P< 0.001*
	Gonotrophic cycle length	11.257, DF=3	P< 0.001*
	# blood meals per gono. cycle	1.567, DF=3	P= 0.224
	Total egg production	10.166, DF=3	P< 0.001*
	Eggs per day	9.909, DF=3	P< 0.001*
	Egg hatch rate	7.944, DF=3	P< 0.001*
	Lifespan	1.289, DF=3	P= 0.282
AKRON	Total # of blood meals	18.582, DF=3	P< 0.001*
	Blood feeding frequency	4.967, DF=3	P= 0.003*
	Lifespan	0.575, DF=3	P= 0.633

a.



b.

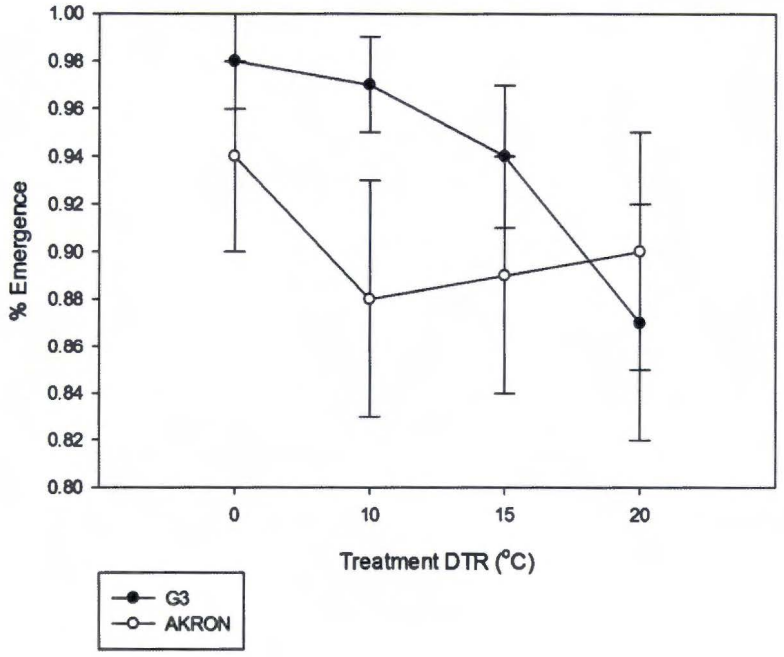


Figure 8. Mean percent and 95% confidence intervals for the pupation (a) and emergence (b) rates by treatment DTR for the G3 and AKRON strains of *Anopheles gambiae*.

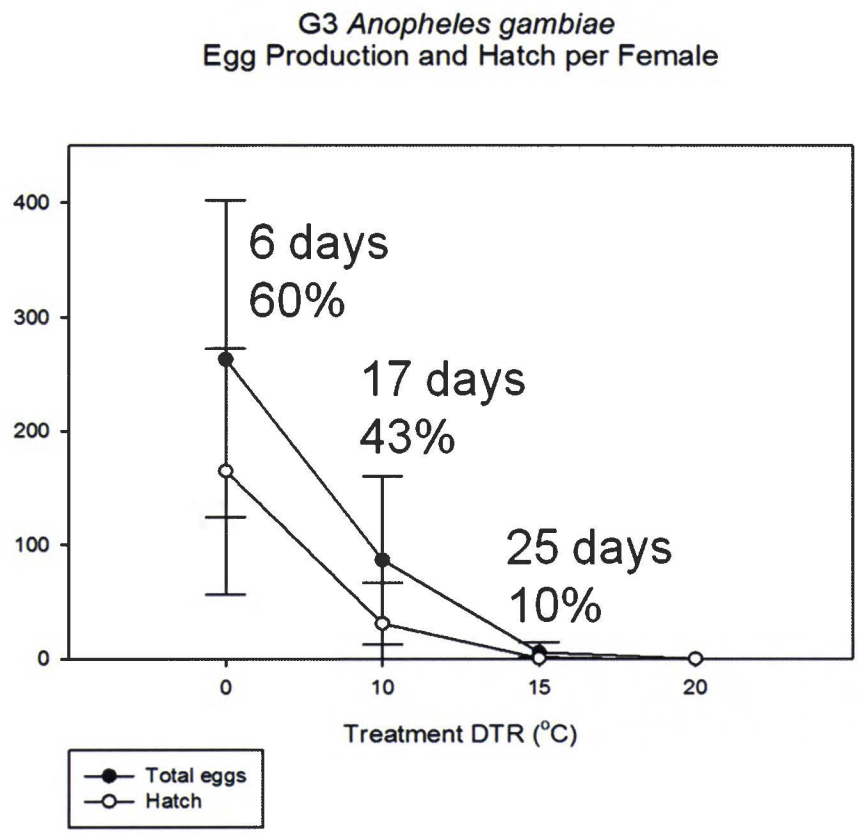


Figure 9. Mean total egg production and hatch from G3 DTR cohorts. Number of days refers to the mean number of days post-emergence until the first oviposition episode for each cohort. Percentage numbers refer to the total percentage of the cohort population that produced eggs.

CHAPTER 5: Influence of Larval DTR Exposure on Adult Insecticide Susceptibility in the Malaria Vector, *Anopheles gambiae* Patton.

ABSTRACT

Insecticide resistance is commonly thought to be the result of physiological and/or behavioral modifications that lead to lack of target site binding, increased detoxification activities, increased sequestration, or avoidance of contact with a compound. Several environmental variables are known to influence adult susceptibility to insecticides, including larval nutritional status and water quality. However, the influence of the thermal environment during larval development has never been considered. Here we report that larval exposure to diurnal temperature fluctuations can influence adult susceptibility to malathion in two strains of the malaria vector, *Anopheles gambiae*, the G3 type strain and the propoxur resistant AKRON strain. High diurnal temperature range (DTR) exposure during larval development resulted in a significant increase, up to a two-fold, in LD_{95} concentrations for the G3 strain and a non-significant 10-20% increase in the AKRON strain. The larval thermal environment had no significant effect on the susceptibility of either strain to propoxur or permethrin. Despite the effects of larval DTR exposure on adult insecticide susceptibility to malathion, changes in susceptibility were not enough to have potential effects on the efficacy of field application rates.

INTRODUCTION

The use of insecticides has long been a cornerstone of vector management strategies. Early successes in crop pest management strategies enticed the World Health Organization (WHO) to initiate the first malaria eradication campaign in 1955. Since that time, the development of resistance to several classes of insecticides employed in control

strategies has been an ongoing issue (16, 30, 33). Resistance, or a decrease in the degree of susceptibility to a given concentration of an insecticide, is influenced by multiple factors. Most commonly, resistance is thought to be solely the result of physiological and/or behavioral modifications that lead to lack of target site binding, increased detoxification activities, increased sequestration, or avoidance of contact with the chemical (16, 30, 60). Also, it is generally accepted that the presence of insecticide resistance imposes fitness costs in a pesticide-free environment. Despite this, relatively little has been reported on the actual costs imposed on a resistant phenotype (3). Insecticide resistance costs currently recognized include reductions in pre-imaginal survival (9, 22), decreased fecundity (25), reduced longevity (3, 13), mating competition costs (10), and increased predation costs (11). Additionally, Rivero et al. (58) showed that, on average, resistant *Cx. pipiens* emerged as adults with 30% less energetic reserves than susceptible mosquitoes. Interestingly, this cost was incurred during metamorphosis, as there was no difference in energetic reserves among resistant and susceptible fourth instar larvae. The quantity of energetic reserves accrued during larval development has also been suggested to influence several factors that determine vector competence and vectorial capacity (59).

Coustau et al. (21) noted that, while some authors presented evidence of resistance associated fitness costs, others were unable to detect a cost. They suggested that resistance costs might only become apparent under a specific set of environmental conditions. Also, while the genetic determinants of insecticide resistance are known (14; 26, 69), the plasticity of phenotypic expression has not been examined. Bourguet et al. (14) did study the plasticity of resistance allele dominance and found that the dominance

of resistance expression (due to an insensitive acetylcholinesterase allele in *Cx. pipiens*) was dependent on the environmental variables examined: larval density, container depth, and daylight length. They showed that the expression of resistance was associated with more demanding environments. In other words, resistance heterozygotes expressed a resistant phenotype only under optimal rearing conditions. Given this, it is reasonable to assume that abiotic environmental variables could influence the cost and expression of insecticide resistance in a population.

The general consensus is to view insecticide resistance in terms of a trait that is both possessed and expressed or not. However, it is possible that biotic and abiotic factors could influence the degree of susceptibility of mosquitoes to a specific insecticide. Some of these influences are relatively intuitive. For instance, larval development in the presence of adequate nutritional resources may allow developing larvae to allocate more resources to detoxification mechanisms, ultimately yielding adults better equipped to detoxify xenobiotics. The "silver spoon" hypothesis posits that larvae developing in the presence of abundant nutritional resources produce healthier adults better able to cope with the vagaries of an unpredictable environment (24). In support of this theory Oliver and Brooke (49) showed that larval nutritional status can influence adult susceptibility to DDT in laboratory strains of *Anopheles arabiensis* Patton. Adult nutritional status (blood-fed, sugar-fed, or starved) has also been shown to influence behavioral responses to insecticide exposure (63).

Other parameters, such as water quality and population age structure, may also have an effect on the expression of insecticide resistance. Tene Fossog et al. (64) suggested that water quality parameters influence larval susceptibility to pyrethrins but

the influence of changing kdr allele frequencies in the source populations could not be ruled out. Several laboratory studies have shown a decrease in phenotypic resistance in older mosquitoes (31, 56) and age since emergence of adult *Anopheles gambiae* Patton has been shown to influence susceptibility to deltamethrin. permethrin, malathion, DDT, and propoxur (19). In essence, Chouaibou et al. (19) showed that older mosquitoes were more susceptible to the tested insecticides than younger mosquitoes.

Ambient temperature could also have the ability to affect susceptibility to insecticide exposure considering that it has been shown to influence life-history traits of vector populations in general (4, 39, 57). Ambient temperature has been shown to have a major influence on vector competence through alteration of interaction dynamics between pathogen and host. For example, Westbrook et al. (70) showed that rearing *Ae. albopictus* at 18°C produced larger females that were six times more likely to be infected by Chikungunya virus than when reared at 32°C. Additionally, Watts et al. (68) showed that, while *Ae. aegypti* could become infected with dengue virus (DEN-2) at any temperature from 20°C to 35°C, only those mosquitoes held at 30°C or higher were able to subsequently transmit the virus to monkeys, regardless of infectious dose. However, the influence of ambient temperature on vector/pathogen interactions is not consistent across systems. For instance, higher temperatures increase infection rates of Rift Valley fever virus in *Cx. pipiens* (67) while decreasing infection rates of Eastern Equine Encephalitis virus in *Cx. tarsalis* (36).

The influence of ambient temperatures during larval development on insecticide resistance is scarcely considered. Nayak and Collins (48) showed that treatment temperature accounted for 75% of the variability in time to population extinction for a

phosphine resistant psocid; that is, temperature being more important than concentration of phosphine used. Kikankie et al. (34) evaluated the effect of ambient temperature on the use of an entomopathogenic fungus for control of resistant and susceptible *An. arabiensis*. While they focused on the viability of the fungal agent at different temperatures, their results showed that both resistant and susceptible strains of *An. arabiensis* exhibited significantly lower fungus induced mortality at lower ambient temperatures (21°C vs. 25°C). At a more physiological level, Yan et al. (71) showed that a single-copy *Apis cerana* Fabricius glutathione S-transferase gene transcript, involved in oxidative stress protection, could be significantly up-regulated during exposure to thermal stress.

Perhaps a more important consideration than ambient temperature alone is that of daily temperature fluctuations, or diurnal temperature range (DTR). The influence of DTR on pathogen-transmission systems has only recently been addressed. Lambrechts et al. (38) showed that *Ae. aegypti* from Thailand were less susceptible to dengue virus infection and exhibited faster mortality at larger DTRs around the same mean ambient temperature. In doing so, they showed that the defining predictive factor for dengue virus transmission in Thailand might not be mean seasonal temperature, or rainfall, but the size of the DTR around the mean daily temperature. Additionally, Paaijmans et al. (53) showed that diurnal fluctuations in temperature reduce the impact of increasing mean temperatures when using a thermodynamic model for malaria development (model parameters 18-28°C). Specifically, they showed that diurnal temperature fluctuations around means >21°C slow parasite development while fluctuations around means <21°C increases parasite development. Expanding on this model, Paaijmans et al. (50)

demonstrated that, at the model extremes, diurnal temperature fluctuations makes transmission possible at lower mean temperatures and reduces transmission at higher mean temperatures more than that predicted by the standard mean temperature models. As a result, current models for predicting malaria transmission risk may underestimate transmission at the fringes of endemic zones, such as in the Highlands of East Africa, while, at the same time, overestimating the risk in warmer portions of endemic zones (50). Despite the increasing focus given to the influence of DTR on adult vector-pathogen transmission dynamics, to my knowledge, the influence of DTR on adult insecticide susceptibility has not been examined.

Just as adult mosquitoes are potentially exposed to diurnal temperature fluctuations in the field, the potential exists for larval development sites to be exposed to the same daily warming and cooling cycles. As such, the thermal environment of the developing larvae should be taken into consideration. The larval thermal environment, however, may be more important than that of adult mosquitoes because adults have the ability to moderate their exposure to daily temperature fluctuations through behavioral avoidance; e.g. responding to changes in microclimactic cues via the search and selection of more suitable resting locations. In contrast, exposure of mosquito larvae to diurnal temperature fluctuations is determined by the physical characteristics and location of the development site. While these fluctuations may be minimal for species that breed in large bodies of water, species that utilize small ephemeral sites such as small puddles, tire ruts, hoof prints, discarded tires and water holding tanks are potentially much more exposed—especially if these locations receive direct sunlight for any portion of the day. *An. gambiae* s.l., the principal vector of malaria in much of Africa, is known to utilize

anthropogenic breeding sites such as tire ruts, hoof prints, and drainage channels (37, 45) as well as natural sites such as rain pools and burrow pits (45). In deforested areas these sites have been shown to be more productive in terms of adult mosquitoes produced (44) than those in forested areas.

Given the exposure of some larval mosquito populations to diurnal changes in temperature, it is surprising that the effect of this exposure on mosquito development has scarcely been addressed. Indeed, there is dearth of information on the temporal temperature profiles of field breeding and development sites in general. Standard practice for all research that involves the use of laboratory-reared mosquitoes utilizes mosquitoes that are reared at a constant temperature. To date, the effect of exposure to diurnal temperature fluctuations during larval development has all but been ignored. In the prior experiments looking at the role of DTR on disease transmission, mosquito vectors were reared under standard rearing conditions with no fluctuations in temperature. In fact, Paaijmans et al. (52) showed that typical anopheline development sites in the field exhibited diurnal temperature fluctuations with a net effect of maintaining mean habitat water temperatures 4-6^{o}C higher than mean ambient temperatures. This difference resulted in faster development times than predicted based on mean ambient temperature alone. The only study to date examining the effect of diurnal temperature fluctuations on the development of mosquito larvae showed a highly female skewed sex ratio at higher temperatures and a lack of correlation between adult body size and rearing temperature (42). Larval exposure to diurnal temperature fluctuations in the field could well explain the results of Tun-Lin et al. (66) who showed

that correlations between wing length and temperature in field populations of *Ae. aegypti* were much lower than that for laboratory-reared mosquitoes.

While not focused on diurnal temperature fluctuations specifically, some authors have shown that short-term exposure of mosquito larvae to thermal stress can affect adult mosquito characteristics. Muturi et al. (47) showed that *Ae. aegypti* larvae reared at 32°C were much more susceptible to infection with Sinbis virus as adults. They also showed that exposure to elevated temperatures during immature development led to a decreased expression of heat shock protein 83 and increased expression of defensin and cecropin in subsequent adults and suggested that this led to gut fauna changes making the mosquitoes more susceptible to infection. Mourya et al. (43) had come to a similar conclusion when they examined the influence thermal stress on *Ae. aegypti* larvae had on adult susceptibility to CHIKV. The only work to date that examines the role larval thermal stress plays in the susceptibility of adults to an insecticide was that of Raghavendra et al. (55) who showed that larval exposure to brief periods of thermal stress resulted in one to three fold increases in adult LT_{50} times to 5% malathion impregnated bed nets.

If brief exposure the thermal stress during the larval stage can influence adult susceptibility to insecticides then it is reasonable to expect that DTR exposure could influence susceptibility as well. Standard practice in resistance screening utilizes field caught mosquito populations reared through the F1 generation under standard laboratory conditions. Understanding the role larval DTR exposure plays in adult susceptibility will help to define the limitations inherent in laboratory assays for insecticide resistance as well as other evaluations that would benefit from further characterization of the laboratory test populations.

In this study we assess the influence larval DTR exposure has on the susceptibility of two strains of *An. gambiae* to three common insecticides; permethrin, malathion, and propoxur. Chemicals were selected to represent the two most common pathways targeted by chemical control. Each chemical has a distinct mode of action but malathion and propoxur target the same pathway. Permethrin targets ion channels in the nerve cell while malathion and propoxur target the aceytlcholinesterase system in the synaptic gaps between nerve cells. The main goal of this study was to determine whether larval DTR exposure could influence adult susceptibility and/or resistance and, if so, whether the direction and magnitude of this effect was consistent despite the resistance status of the strain used. To do this, two strains of *An. gambiae* were used, the G3 strain originally from The Gambia and the knockdown resistant AKRON strain from Benin. Also, the use of multiple insecticides allowed us to test whether any observed effects were non-selective, or if DTR exposure differentially affected susceptibility to insecticides with differing modes of action.

METHODS

Mosquito Strains

Wild type G3 strain susceptible (F240) and carbamate resistant AKRON strain (F5) *An. gambiae* eggs were obtained from the Centers for Disease Control's Malaria Research and Reference Reagent Resource Center (MR4) in Atlanta, GA. Eggs were used to establish laboratory colonies at the USUHS insectaries to serve as source stock for multiple experiments.

Rearing Conditions

All mosquito cohorts were reared using Percival I-36VL incubators (Percival Scientific, Perry IA). Briefly, eggs were hatched under standard rearing conditions (28°C, LD 12:12, RH 80%) and hatched larvae allowed to develop for 24 hrs. Larvae were then separated at the rate of 200 larvae per 18 x 25 cm collection tray containing 900ml dH_2O. Each larval tray was fed a total of 0.42g ground TetraFin® goldfish flakes (Tetra Holding, inc, Blacksburg, VA) over the course of larval development (0.045g on days 1, 3, 4, and 7; 0.12g on days 5 and 6). Trays were skimmed by lightly dragging a kim-wipe across the surface of the water on day 3 and on day 4 an additional 450 ml of dH_2O, warmed to incubator temperature, was added to each tray. Pupae were collected with disposable pipettes and placed in 500 ml emergence cups inside one gallon plastic buckets. Pupae and adults were maintained under standard rearing conditions (28°C, LD 12:12, RH 80%) and provided 10% sucrose cotton pledglets ad libitum. Larval cohorts from each strain were reared under one of four daily temperature treatments (LD 12:12, RH 80%) from 24hrs post hatch to pupation. Temperature treatments consisted of a DTR of 0°C, 10°C, 15°C and 20°C around a mean 28°C. Daily temperature curves (except for the 0°C range) followed a truncated sine wave progression during the light phase (day) and a decaying exponential progression during the dark phase (night). These curves have been found to accurately mimic diurnal variation in soil and air temperature during the course of a solar day (54). One cohort from each strain was reared under each DTR treatment for a total of 8 cohorts for use in the following experiments. Both air and water temperature profiles were monitored with HOBO data loggers (Onset, Cape Cod, MA). Mean daily air temperature ranged from 27.4°C to 28.1°C while mean daily water temperature ranged from 26.7°C to 27.4°C across all treatments. All treatment daily

mean temperatures were within 0.2°C of each other except for those cohorts reared under a 20°C DTR, which were, on average, 0.5°C cooler representing the lower end of the range.

Insecticide Assays

To evaluate the influence larval DTR exposure has on adult mosquito susceptibility to selected insecticides a series of CDC bottle assays were conducted using permethrin, malathion, and propoxur. All insecticide assays were performed using the current MR4 CDC bottle assay protocol (17, 20). Fifteen to 20, 3-7 day old female mosquitoes were separated into one pint holding cups and provided with 10% sucrose pledglets 24 hours before assay start. Assays consisted of five concentrations per chemical. Dosage concentrations were originally calculated in nmol/cm^2 and selected to provide percent mortality counts with enough granularity to allow construction of probit transformed regression lines. All concentrations were then back-transformed to ng/cm^2 to allow easy comparison to standard field application rates. Insecticides were dissolved and diluted in molecular grade acetone and control bottles treated with acetone. Concentrations selected for use with G3 assays were 199.55, 50.08, 12.52, 3.13, and 0.78 ng/cm^2 for permethrin, 4.19, 2.09, 1.47, 1.05, and 0.04 ng/cm^2 for propoxur, and 14.54, 11.23, 7.93, 4.63, and 1.32 ng/cm^2 for malathion. Concentrations for the AKRON strain were 199.55, 50.08, 12.52, 3.13, and 0.78 ng/cm^2 for permethrin, 209.24, 104.62, 26.16, 1.47, and 1.05 ng/cm^2 for propoxur, and 82.59, 49.55, 16.52, 8.26, and 3.3 ng/cm^2 for malathion. Glass bottles (250ml Wheaton) were treated with 1ml of solution, capped and swirled for 5 seconds to ensure treatment of the lid, and then placed on a bottle roller (Wheaton Science Products, Millville, NJ), lid removed, and allowed to dry while rolling

(approximately 5 minutes each). Bottles were then placed under a fume hood with lids off and loosely covered with aluminum foil overnight.

Three pools of 15-20 mosquitoes each were used for testing at each concentration and for each control. All assays were run for 60 minutes with percent knockdown being recorded at 15-minute intervals. Knockdown was defined as any mosquito unable to fly, walk, or rest in a deliberate manner. After 60 minutes all mosquito pools were placed in 1pint holding containers, provided with a 10% sucrose pledglet and held at 28°C, 80% RH, LD: 12/12 for 24 hrs to obtain 24hr mortality counts. When control mortality exceeded 5% the percent mortality for all concentrations in the assay were transformed using abbot's formula (1). If control mortality exceeded 10% mortality data was discarded and the assay was repeated. Mortality data were analyzed using the log-probit regression function in SPSS version 20. Treatments were considered significantly different from each other when the ratios of their $LD_{50/95}$ had confidence limits that excluded one.

RESULTS

While there was some variability in LD_{50} and LD_{95} concentrations between DTR treatments, overall, larval DTR exposure did not have a significant influence on these values (Table 6). No clear trend was seen for permethrin with increasing DTR for either strain. The LD_{95} concentrations for the G3 strain ranged from 105.74 to 146.92 ng/cm^2 while those for the AKRON strain were more variable, ranging from 249.73 to 411.8 ng/cm^2. Results for propoxur were similar for the G3 strain with LD_{95} values ranging from 3.2 to 3.78 ng/cm^2. Being a kdr strain with resistance to propoxur, the AKRON LD_{95} values were much higher, ranging from 956.21 to 5801.26 ng/cm^2. Despite the

large spread between the lowest and highest LD_{95} concentrations for this compound, none of the pairwise comparisons were significantly different from the 0°C control and no trend with increasing DTR was observed. Malathion proved the only exception to this general lack of trend with the G3 strain 15°C and 20°C cohorts exhibiting significantly higher $LD_{50/95}$ values than the 0°C control cohort. As a result, dose-response curves for the G3 cohort exhibited significant shifts to the left for the 15°C and 20°C cohorts (Figure 10). This general trend was also detected in the AKRON strain, with the 15°C and 20°C cohort dose response curves exhibiting a shift to the left compared to that the 0°C and 10°C cohorts, though not significant (Figure 10).

DISCUSSION

The AKRON strain was more resistant than the G3 strain to all compounds tested. This strain is known for its resistance to propoxur as was evident from the results presented here. Interestingly, for propoxur, the difference between the AKRON and susceptible G3 strain increased as you moved up the percent mortality curve. The AKRON LD_{50} values were roughly 8-fold higher than those of the G3 strain while the LD_{95} values were 300 to 1700-fold higher, depending on the cohort compared. Over the course of the assays initial knockdown time appeared to be similar for both the G3 and AKRON strains. However, a smaller proportion of mosquitoes were initially knocked down in the AKRON strain as compared to the G3 strain. Furthermore, the number of AKRON mosquitoes knocked down remained constant throughout the course of exposure, unlike the G3 strain, which exhibited the standard increased knockdown response per unit time exposed. This suggests that some individuals in the AKRON strain are just as susceptible to knockdown as are the G3 strain mosquitoes but those that

aren't knocked down initially are able to avoid knockdown altogether. It seems this tendency for all or nothing knockdown at the individual level contributed to the large amount of variability in the results between the AKRON cohorts. Additionally, AKRON mosquitoes that were knocked down had a greater tendency to survive the 24 hour holding period than did knocked down G3 mosquitoes. This trend of increased difference between LD values as you moved up the percent mortality curve was also evident in the malathion data, though not nearly as dramatic.

As expected, some cross-resistance to the organophosphate malathion was observed in the AKRON strain. However, unlike propoxur, increased DTR appeared to decrease susceptibility to malathion. Both the 15°C and 20°C cohorts from the G3 strain exhibited an almost two-fold increase in the $LD_{50/95}$ concentrations compared to the 0°C control and 10°C cohorts. A slight increase in $LD_{50/95}$ concentrations among the 15°C and 20°C cohorts in the AKRON strain was also detected. This is consistent with what Raghavendra et al (55) reported for *Anopheles stephensi* Liston mosquitoes where brief exposure to thermal shock as larvae resulted in one to three-fold increases in LT_{50} times when exposed to bed nets treated with 5% malathion. Despite this, the decreased susceptibility observed in the 15°C and 20°C cohorts would not be enough to affect the field efficacy of malathion when applied at the WHO recommended rate of 2 g/m^2. Also, given that the reproductive success of the cohorts exposed to 15°C and 20°C DTRs fell to zero (Chapter 4), less susceptible mosquitoes from field development sites with such a thermal profile would quickly drive the population to extinction without the need for chemical control.

Somewhat surprisingly, AKRON cohorts were less susceptible to permethrin than the G3 cohorts with $LD_{50/95}$ values roughly three-fold higher than those for the G3 cohorts. Unlike for propoxur and malathion, the magnitude of the difference between the AKRON and G3 LD concentrations remained constant throughout the percent mortality curve. This difference in susceptibility between the G3 and AKRON strains suggests that the AKRON strain may exhibit some cross resistance to permethrin as well. Since the resistance to propoxur exhibited by the AKRON strain is due to a point source acetylcholinesterase mutation, it is unlikely that this mutation also provides a level of cross resistance to permethrin, which targets sodium ion channels along the nerve axon. It is possible that there is also metabolic resistance that is functioning in conjunction with the acetylcholinesterase mutation that provides some cross resistance to permethrin via increased detoxification of the compound. Some evidence for this dual mechanism approach to resistance has been suggested for *Ae. aegypti* populations from Martinique (41). In fact, pyrethroid resistance has been shown to be the result of both target site insensitivity (west kdr pyrethroid resistance mutation) and over-expression of detoxification genes such as cytochrome P450 enzymes in *An. gambiae* populations from the agricultural area of Akron, Benin (23); the source of the carbamate resistant AKRON strain. Given this, it is quite feasible that multiple resistance mechanisms are present in the AKRON strain.

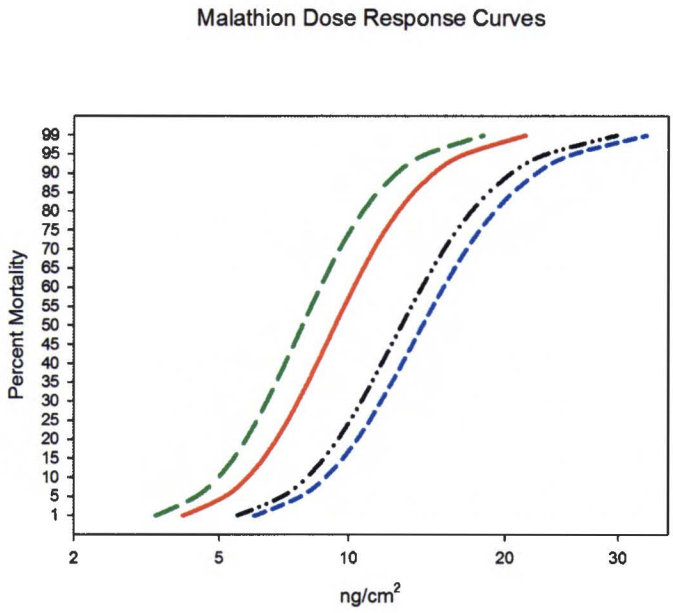
Based on the work presented here, it appears that larval exposure to differing thermal regimens has a negligible effect on the susceptibility of both susceptible and resistant strains of *An. gambiae* to selected compounds from three major insecticidal classes. With the exception of the organophosphate, malathion, no clear trend was

detected with increasing larval DTR exposure. For malathion, increasing DTR led to decreases in adult susceptibility as evidenced by higher LD_{50} and LD_{95} values. This trend was detected in both the susceptible G3 and carbamate resistant AKRON strain but was only significant in the G3 strain. Additionally, the AKRON strain exhibited higher LD_{50} and LD_{95} values than the G3 strain suggesting some possible cross-tolerance to permethrin as well. However, the AKRON LD_{95} values for permethrin ranged from 3-4 mg/m^2, well below the WHO recommended 20-30 mg/m^2 for indoor residual spray and bed net applications of pyrethroids. Whether these differences were due to increased metabolic resistance in the AKRON strain or simply due to differences in source population variation could not be determined. It is also possible that colony age was a contributing factor to these results. The G3 strain stock was started from generation F240, whereas the AKRON strain was only recently established, with starting stock for the above experiments from the F4 and F5 generations. Further work is needed to isolate cause and effect with respect to DTR exposure and insecticide susceptibility. Controlling for source stock and colony age would enable better detection of any susceptibility by DTR trends that we were unable to detect due to experimental constraints. Additionally, whether the same trends would be detected using insecticides applied to field surfaces remains to be seen. As a result, differences in susceptibility reported here, while well below recommended field application rates, could very well influence field efficacy when active ingredients are applied to non-sterile surfaces exposed to multiple environmental variables.

Table 6. LD_{50} and LD_{95} values calculated from dose-response curves using log-probit regression methods for all chemicals tested across strains. Sample sizes for all curve estimations were 270-360 mosquitoes per cohort tested. Significance (*) was determined by comparing the ratios of LD_{50} and LD_{95} values between the 0°C control assays and each treatment. If the upper and lower 95% confidence intervals did not include one the difference between control and treatment was deemed significant. Dosage units for the $LD_{50/95}$ values are in ng/cm^2.

		Permethrin		Propoxur		Malathion	
Strain	**Treatment**	**LD_{50}**	**LD_{95}**	**LD_{50}**	**LD_{95}**	**LD_{50}**	**LD_{95}**
G3	**0**	18.67	130.22	1.14	3.49	9.33	16.91
	10	15.16	105.74	1.12	3.42	7.91	14.32
	15	21.07	146.92	1.05	3.2	14.15	25.64*
	20	19.16	133.59	1.24	3.78	12.87	23.31*
AKRON	**0**	60.34	303.09	8.5	2919.51	11.3	37.25
	10	77.95	391.55	6.75	2316.68	13.21	43.54
	15	49.72	249.73	16.9	5801.46	17.09	56.31
	20	81.98	411.8	2.79	956.21	14.73	48.55

a.



b.

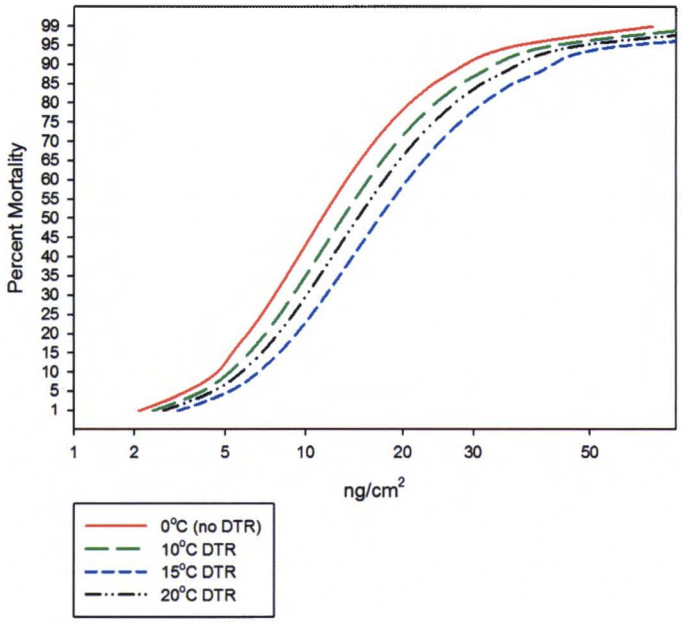


Figure 10. Twenty four hour post-exposure malathion dose-response curves for the G3 (a) and AKRON (b) strains of *Anopheles gambiae*. Values on the x-axis represent bottle malathion AI residuals in ng/cm^2. The y-axis is the percent mortality predicted by the probit model.

CHAPTER 6: Conclusion

Given that a large amount of what is known about adult mosquito biology was gleaned from captive-reared colonies exposed to constant rearing temperatures, it is important to understand the impact that daily changes in the thermal environment may have on the traits that are measured. It is obvious from the work presented here that the parameters of the larval thermal environment can influence the expression of traits in the adult mosquito. Furthermore, it is apparent that diurnal temperature fluctuations in the larval environment affect mosquitoes in species specific ways. As shown in chapter 4, increasing DTR in the larval environment has a profoundly negative effect on the reproductive success of *An. gambiae* adults. This occurs so much to the extent that egg production ceases altogether when individual mosquitoes are exposed to a 20°C DTR during larval development. This may help explain why An. gambiae do not utilize container breeding habitats in the wild. While the larvae are capable of surviving under a 20°C DTR, albeit at a lower pupation rate, adult females seem incapable of producing eggs. They continue to take and digest blood meals but oviposition behavior, for some reason, is interrupted.

In contrast, the results obtained for *Ae. aegypti* were remarkably different (Chapter 2). For this species, egg production was generally not affected by larval DTR exposure. In fact, exposure to mild DTRs (10-15°C) during larval development in the BZ strain led to an increase in egg production in the adult stage. Similar results were also reported for *Ae. aegypti* from Thailand (18). However, this trend was not detected among the TH stain tested here. Our results showed a lack of influence on egg production except at the extreme (20°C), where a decrease in egg production was

detected. This decrease in egg production with a large DTR was consistent with that reported by Carrington et al. (18). This suggests that there may be some variability in the response to DTR exposure between geographic populations within the same species.

By comparing other traits, it was observed that this variability can be trait dependent as well. For example, increasing DTR resulted in slightly elevated pupation rates in the BZ strain and decreased pupation rates in the TH strain of *Ae. aegypti*. Despite this, increasing DTRs resulted in reduced adult sucrose survival times among male BZ cohorts and increased survival times for male TH cohorts. Female adult sucrose and bloodfed survival times were generally not affected by DTR for either strain. In general, larval exposure to DTRs of different magnitude only affected measured adult characteristics at the larger DTRs tested for the TH strain. In the majority of traits examined here, only pupation rates in the TH cohorts showed a clear trend with increasing DTR. For other variables, the differences were mostly between 0-10°C and the 15-20°C TH cohorts. For the BZ cohorts, conversely, differences were mostly between the 0°C control cohort and the 10-20°C cohorts.

For *An. gambiae*, pupation and emergence rates decreased with increasing DTR for both the G3 and AKRON strains. However, the carbamate resistant AKRON strain exhibited pupation and emergence rates that were consistently lower than those of the susceptible G3 strain regardless of DTR. In this respect, it appears that possession of the ACE-1 mutation in the AKRON strain may impair the ability of this strain to meet the physiological requirements for pupation and emergence regardless of the larval thermal environment. However, AKRON strain mosquitoes that reached adulthood exhibited longer survival times than those observed in the G3 strain. Also, increasing DTR resulted

in increased sucrose survival rates for the AKRON strain while causing a decrease in survival rates in the G3 strain. Despite the increased survival in the AKRON strain, however, egg production was almost zero, even for the 0°C cohort. This lack of egg production in the AKRON strain may have influenced the outcomes of other variables as well. For instance, the G3 cohorts took more blood meals than the AKRON cohorts despite having decreased survival rates in the 0°C and 10°C cohorts.

It is apparent that larval DTR exposure can alter the outcome of epidemiologically important traits. The magnitude and direction of these effects also appear to be not only species specific, but population specific, as well. Given this, several variables that influence a population's vectorial capacity could be affected by DTR exposure, thus altering the estimate of a populations' ability to propagate a pathogen of interest. Common variables that have the potential to be affected by temperature are blood feeding frequency, survival, population density, and vector competence. While the conventional approach is to assume vector competence and vectorial capacity is temperature independent, Paaijmans et al. (51) has shown that this assumption leads to erroneous vectorial capacity estimates by temperature which, in the case of malaria transmission, overestimates transmission probability at warmer temperatures. As a result, the use of models that utilize mean daily, monthly, or annual temperatures may provide inadequate information and lead to erroneous conclusions about the transmission dynamics of interest.

As with the life history traits measured, exposure to diurnal temperature fluctuations during larval development had variable results on insecticide susceptibility that were species, and sometimes strain, specific. For both *Ae. aegypti* strains tested, a

small DTR of 10°C during larval development decreased susceptibility to both propoxur and malathion in the adult stage while DTRs larger than 10°C increased adult susceptibility. However, for the *An. gambiae* strains, increasing DTR had limited effects on their susceptibility to propoxur while causing a decrease in susceptibility to malathion in both strains. This was consistent with what Raghavendra et al. (55) reported for *An. stephensi* mosquitoes, where brief exposure to thermal shock as larvae resulted in one to three-fold increases in LT_{50} times when exposed to bed nets treated with 5% malathion. For susceptibility to permethrin, the direction trend with increasing DTR was dependent on the *Ae. aegypti* strain used while, for both *An. gambiae* strains, no trend was detected. Belize *Ae. aegypti* mosquitoes showed a steady increase in susceptibility to permethrin out to the 20°C DTR treatment while the TH mosquitoes exhibited a decrease in susceptibility at DTRs of 15°C and above.

Even though larval DTR exposure had an influence on adult susceptibility in some of these trials, none of the differences were such that field application rate efficacies would be affected. However, it is important to note that residuals were applied to sterile, non-porous, glass surfaces under controlled temperature and humidity and not to field substrates under field conditions. The amount of active ingredient actually available for contact by mosquitoes in the field may be much less then field application recommendations due to possible interactions with external variables, such as organic compounds and UV light. Under this assumption, it remains to be seen whether DTR exposure during larval development can actually reduce or increase the effectiveness of standard application rates in the field.

The work presented here represents the beginning of an examination on how the thermal parameters that occur during larval development may influence adult characteristics of commonly studied disease vectors. This work was prompted by the frustration of trying to sustain a struggling An. gambiae colony in an insectary that lacked standard climate control. To this end I believe I have found an answer—rearing of *An. gambiae* larvae under a regimen that does not control daily temperature fluctuations results in decreased egg production in the adults. While, on the surface, this may seem like an obvious justification for the use of constant rearing temperatures, it also opened up the possibility that there is an environmental parameter whose ramifications, to date, have remained to be considered. While work has been done on how different mean temperatures affect development of various mosquito species, it was not until 2013 (concurrently and independently of this work) that a study designed specifically to examine daily temperature fluctuations during larval development was completed (18).

To this end this dissertation opens up many new avenues for future research. Despite showing a clear trend by DTR for some traits, there is clearly a need to continue to evaluate the biological relevance of these parameters regardless of species. Basic work, such as documenting the actual thermal environment of mosquito breeding sites in the field, has yet to be completed. Thermal parameters of breeding habitats will vary depending on the preferences of the mosquito species under examination. Documenting the daily thermal parameters in preferred breeding habitats of common disease vectors will allow modelers to determine whether utilization of mean temperature data is an appropriate strategy or not. Furthermore, once these parameters are documented, it will

allow for the development of more realistic experiments to address whether these factors have an epidemiologically relevant influence on traits of interest.

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